CONTEMPORARY CANCER RESEARCH

DNA Damage and Repair

Volume III: Advances from Phage to Humans

EDITED BY

Jac A. Nickoloff

Merl F. Hoekstra



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DNA Damage and Repair

Volume 3

Advances from Phage to Humans

Edited by

Jac A. Nickoloff

University of New Mexico, Albuquerque, NM

and

Merl F. Hoekstra

Qbiogene, Carlsbad, CA

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Preface

Even before we completed Volumes I and II of *DNA Damage and Repair* in 1998, three facts made it very clear that a third volume would be necessary. First, despite our best attempts at providing comprehensive coverage of this rather large and rapidly expanding field, we were unable to identify authors for several important topics. Volume III: *Advances from Phage to Humans* thus fills some of the gaps in the previous volumes, including DNA repair in bacteriophage and *Drosophila*, and the role of DNA repair in the generation of immune diversity. Second, the DNA repair field continues to grow explosively, and several topics needed updating soon after the first volumes were published. Such topics include the role of homologous recombination in mammalian cells, and the new biochemistry and cell biology of DNA double-strand break repair, which has provided key information about protein function in this important biological process. Third, as might be expected from such an active field, there are several new areas of research that were not even imagined prior to 1998, including the finding that proteins involved in nonhomologous end-joining were also involved in gene silencing and telomere function, and the discovery that the breast cancer susceptibility genes, *BRCA1* and *BRCA2*, have important roles in several aspects of DNA repair.

The DNA repair field grew from basic studies in genetics and cell biology. These approaches are increasingly complemented by biochemical approaches that provide detailed descriptions of complex processes at the molecular level and identify functional interactions among the various proteins involved in each repair pathway. Although early work provided provocative hints that DNA repair processes were conserved from bacteria to higher eukaryotes, a full appreciation of this conservation was not possible until many more genes were isolated and sequenced, and their gene products characterized at the biochemical level. This area of research has in turn led to the understanding that functions carried out by a single protein in prokaryotes are often performed by several related proteins in eukaryotes, and that these protein family members are often found in multi-subunit complexes. Another new development in the field is that seemingly distinct DNA repair processes, such as mismatch repair and nucleotide excision repair, show functional overlap, particularly at the level of lesion recognition. Such overlap suggests that DNA repair processes form a complex network. It is likely that this network enables cells to respond appropriately to different quantities and qualities of DNA damage. As we stand on the threshold of the "New Age of Functional Genomics and Proteomics," it is clear that the next level of understanding will be a molecular description of DNA repair networks in various cell types, and how these networks produce the various cellular responses to different types of DNA damage. Of course, a discussion of the future of DNA repair research begs the question:

Will there there be a need for Volume IV? The answer of course is yes, but just when this task will be undertaken (and by whom) is not yet clear.

We thank all of the contributors for their considerable time and effort to produce the high-quality texts, and for their assistance in the development of the chapter titles and in reviewing draft manuscripts. We also thank our many colleagues, both within and outside our laboratories, for their continued support. And we again thank our families for their patience and understanding: Denise, Jake, Ben, Courtney, Debra, Brad, Lauren, Brielle, and Alexa.

Jac A. Nickoloff
Merl F. Hoekstra

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Contributors

COLIN A. BILL • *Department of Molecular Genetics and Microbiology, University of New Mexico School of Medicine, Albuquerque, NM*

SUSAN M. BAILEY • *Bioscience Division, Los Alamos National Laboratory, Los Alamos, NM*

CAROL BERNSTEIN • *Microbiology and Immunology, College of Medicine, University of Arizona, Tucson, AZ*

HARRIS BERNSTEIN • *Microbiology and Immunology, College of Medicine, University of Arizona, Tucson, AZ*

MARK A. BRENNEMAN • *Department of Molecular Genetics and Microbiology, University of New Mexico School of Medicine, Albuquerque, NM*

DEBRA A. BRESSAN • *Laboratory of Genetics, University of Wisconsin, Madison, WI*

JAYNE S. DANSKA • *Program in Developmental Biology, Hospital for Sick Children and Department of Immunology, University of Toronto, Toronto, Ontario, Canada*

TROY FIDDLER • *The Vollum Institute and Department of Molecular and Medical Genetics, Oregon Health Sciences University, Portland, OR*

CARLOS C. FLORES • *Laboratory of Genetics, University of Wisconsin, Madison, WI*

PAUL S. FREEMONT • *Imperial Cancer Research Fund Laboratories, Lincoln's Inn Fields, London, UK*

EDWIN H. GOODWIN • *Bioscience Division, Los Alamos National Laboratory, Los Alamos, NM*

MICHAEL A. GORMAN • *Imperial Cancer Research Fund Laboratories, Lincoln's Inn Fields, London, UK*

CYNTHIA J. GUIDOS • *Program in Developmental Biology, Hospital for Sick Children and Department of Immunology, University of Toronto, Toronto, Ontario, Canada*

JAMES E. HABER • *Rosenstiel Center and Department of Biology, Brandeis University, Waltham MA*

IAN D. HICKSON • *The Institute of Molecular Medicine, Imperial Cancer Research Fund Laboratories, University of Oxford, Oxford, United Kingdom*

JING HUANG • *The Vollum Institute and Department of Molecular and Medical Genetics, Oregon Health Sciences University, Portland OR*

MARIA JASIN • *Cell Biology and Genetics Program, Sloan-Kettering Institute and Cornell University Graduate School of Medical Sciences, New York, NY*

Teresa Johnson-Pais • *Department of Pediatrics, The University of Texas Health Science Center, San Antonio, TX*
Alan R. Lehmann • *MRC Cell Mutation Unit, Sussex University, Falmer, Brighton, UK*
Eva Y.-H. P. Lee • *Institute of Biotechnology and Department of Molecular Medicine, University of Texas Health Sciences Center at San Antonio, TX*
Richard S. Maser • *Laboratory of Genetics, University of Wisconsin, Madison, WI*
Julianne Meyne • *Bioscience Division, Los Alamos National Laboratory, Los Alamos, NM*
Martin Nemec • *Program in Developmental Biology, Hospital for Sick Children and Department of Immunology, University of Toronto, Toronto, Ontario, Canada*
Jac A. Nickoloff • *Department of Molecular Genetics and Microbiology, University of New Mexico School of Medicine, Albuquerque, NM*
Lauryl M. J. Nutter • *Program in Developmental Biology, Hospital for Sick Children; Department of Immunology, University of Toronto, Toronto, Ontario, Canada; John Radcliffe Hospital, Headington, Oxford, United Kingdom*
Noreen E. O'Regan • *Biology Department, Northeastern University, Boston, MA*
Elizabeth Ostermeyer • *The Vollum Institute and Department of Molecular and Medical Genetics, Oregon Health Sciences University, Portland, OR*
Chrystal K. Palaty • *Program in Developmental Biology, Hospital for Sick Children and Department of Immunology, University of Toronto, Toronto, Ontario, Canada*
John H. J. Petrini • *Laboratory of Genetics, University of Wisconsin, Madison, WI*
Galina Petukhova • *Institute of Biotechnology and Department of Molecular Medicine, University of Texas Health Sciences Center at San Antonio, TX*
Steven J. Sandler • *Department of Microbiology, University of Massachusetts, Amherst, MA*
Phyllis R. Strauss • *Biology Department, Northeastern University, Boston, MA*
Patrick Sung • I*nstitute of Biotechnology and Department of Molecular Medicine, University of Texas Health Sciences Center at San Antonio, San Antonio, TX*
Elaine M. Taylor • *MRC Cell Mutation Unit, Sussex University, Brighton, UK*
Mathew J. Thayer • *The Vollum Institute and Department of Molecular and Medical Genetics, Oregon Health Science University, Portland, OR*

1
DNA Repair in Bacteriophage

Carol Bernstein and Harris Bernstein

1. INTRODUCTION

Starting with the very first studies on DNA repair in 1947, most of the earliest work, involving recombinational repair, photoreactivation, and excision repair, were carried out with bacteriophage (phage) T4 (reviewed in *4,6*). Repair processes in phage appear to be similar to DNA repair processes in other organsims, and genes necessary for DNA repair in phage, such as phage T4 genes *denV* and *uvsX,* are homologous to repair genes in bacteria and eucaryotes (*see* Sub heading 7.). Thus, study of DNA repair processes in phage illuminates the mechanisms and adaptive functions of similar, but often more complex, processes in bacteria and eucaryotes.

The various phages differ in the extent to which they depend on host functions for repair of DNA damage. Two well-studied examples at opposite ends of the spectrum are phage T4 and phage λ. Phage T4 depends largely on gene products encoded by the phage genome itself, while phage λ depends to a great extent on bacterial host functions. In this review we have specifically focused on repair processes encoded by phage genomes. DNA repair processes of phage reliant on bacterial host gene products are ordinarily considered in the context of bacterial repair processes.

2. PATHWAYS OF REPAIR

The first two repair processes discussed in this section, DenV associated base-excision repair and photoreactivation, are specific to ultraviolet (UV)-induced pyrimidine dimers. The next repair process discussed, multiplicity reactivation (MR), is a general recombinational repair process that can act against a wide variety of lesions. It is highly effective against lesions caused by reactive oxygen species, which are likely to be important under natural circumstances. Double-strand break (DSB) repair also appears to be a recombinational repair process similar or identical to MR. Mismatch repair in phage T4 is a specialized process for resolving mismatched bases that can arise in heteroduplex DNA formed during recombination. In post-replication recombinational repair (PRRR), a DNA molecule with a single-strand damage replicates and forms two daughter molecules, one of which has a single-strand gap opposite the damaged template strand. This gap can then be filled in by recombination with the intact daughter chromosome. This process does not directly remove the damage, but rather allows

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accurate bypass of the damage. The Luria-Latarjet effect appears to be a consequence of both base-excision repair and recombinational repair. Error-prone repair may reflect lower accuracy of the DNA synthesis associated with a recombinational repair process compared to the DNA synthesis associated with normal chromosome replication. Finally, replication repair occurs by an unknown mechanism that appears to be independent of base-excision repair and recombinational repair.

2.1 Base-Excision Repair

In phage T4, excision repair processes appear to be limited to the pathway of base-excision repair of UV-induced pyrimidine dimers in which the first enzyme to act is DNA endonuclease V (DenV, the product of T4 gene *denV*). T4 DenV is a bifunctional enzyme with both a pyrimidine-dimer specific glycosylase activity and an apurinic/apyrimidinic (AP) lyase activity. The pathway of DenV mediated base-excision repair has been extensively studied (the early work is reviewed in ref. [6] and more recent studies are reviewed by Valerie [67]). The first step in the pathway is the cleavage of the N-glycosyl bond at the 5′-side of the pyrimidine dimer by the action of the glycosylase activity of the DenV protein, resulting in an AP site. This is followed by incision of the sugar phosphate backbone on the 3′ side of the AP site by the AP lyase activity of DenV. Then a second incision on the 5′ side of the AP site is made, presumably by the AP lyase activity of T4 Gp30 DNA ligase (*11;* Subheading 3.11.). The next step is thought to be removal of the remaining portion of the pyrimidine dimer by the 5′→3′ exonuclease activity of the host *Escherichia coli* DNA polymerase I. Concomitant with this removal, the single-strand gap created by the removal is filled in by the polymerizing activity of DNA polymerase I. The average patch size is between 4 and 7 nucleotides. Repair is completed by DNA ligase (Gp30), which seals the last phosphodiester bond.

2.2. Photoreactivation

Photoreactivation is the enzyme-mediated light-dependent monomerization of pyrimidine dimers, resulting in repair of DNA. In T-even phage, photoreactivation is likely owing to a host enzyme, since this process occurs in phage T2 as early as 10 seconds after adsorption. Photoreactivation appears to be a major mode of repair of UV-induced lethal damage in phage T4 (reviewed in *4,6*). Photoreactivation also has been studied in phages that infect *Vibrio cholerae.* UV-induced DNA damage in phages of different morphological and serological groups is efficiently photoreactivated *(3,55,61).*

2.3. Multiplicity Reactivation

When phage are treated by a DNA damaging agent (causing reduced survival), it is often found that allowing multiple phage to infect each cell gives a level of survival of infective centers that is substantially greater than would be expected from the presence of multiple independent targets. This phenomenon, referred to as multiplicity reactivation (MR), implies that when more than one phage chromosome are present within a host cell, a DNA repair process is available that is not active when there is only one phage chromosome per cell. A substantial amount of evidence (reviewed in *4,6*) indicates that MR is a recombinational repair process. First, recombination is implicated because, by definition, the process requires two or more chromosomes. Second, for phage T4 damaged by UV, nitrous acid (HNO_2) or mitomycin C (MMC), MR depends

on several gene functions required for normal levels of spontaneous recombination (e.g., Gp32, Gp46, Gp47, UvsX, and UvsY). Third, under conditions where MR occurs, the frequency of genetic recombination increases. Fourth, a mutation in gene *46* or *47* reduces or eliminates both MR of HNO_2-damaged phage and the increased genetic recombination caused by HNO_2 damage. Hydrogen peroxide (H_2O_2) and its free-radical product, the hydroxyl radical (OH•) are ubiquitous major sources of DNA damage in living organisms. The lethal DNA damages caused by H_2O_2 in phage T4 were repaired by MR more efficiently than most other types of damages studied *(14).* MR has also been found in phage T7 inactivated by methyl methanesulfonate (MMS) *(35).*

2.4. Double-Strand Break Repair

DSBs are produced by X-rays and by the decay of incorporated ^{32}P. Other lesions are also produced in both cases, but inactivation of the phage is correlated with DSBs (reviewed in *43*). Thus, the MR observed after damage caused by either ^{32}P decay or X-irradiation is inferred to involve DSB repair. Other studies suggest that a special protein-linked form of DSB can also be recombinationally repaired. The anti-tumor acridine *m*-AMSA inhibits the growth of phage T4 by targeting the phage-encoded type II DNA topoisomerase *(31).* Topoisomerase-deficient mutants were shown to be resistant to *m*-AMSA, indicating that *m*-AMSA inhibits growth by blocking the cleavage complex from being further processed, rather than by inhibiting enzyme activity. The inhibitor traps a reaction intermediate consisting of a covalent protein-DNA complex in which both 5′ ends of a DSB are linked, via phosphotyrosine bonds, to the topoisomerase. This complex appears to be subject to recombinational repair on the basis of evidence that mutations in genes necessary for recombination (*uvsX, uvsY, 46/47,* and *59*) each increase sensitivity to *m*-AMSA, and *m*-AMSA stimulates recombination during T4 infection *(54).* Sensitivity to *m*-AMSA was also increased by a mutation in *uvsW (72).* Furthermore, a mutation in the *rnh* gene (RNase H and 5′ to 3′ exonuclease) also increased sensitivity to *m*-AMSA, and to UV as well, suggesting that the Rnh protein may be involved in recombinational repair *(72).*

Topoisomerase I (TOP1)-mediated DNA damage induced by camptothecin in the presence of active transcription was studied using purified calf-thymus TOP1 and phage T7 RNA polymerase *(73).* Transcription elongation processed reversible TOP1-camptothecin-DNA cleavable complexes into irreversible strand breaks on the template, but not on the nontemplate strand, within the transcribed region. This suggests a model in which collision between the TOP1-cleavable complexes located on the template strand and the elongating RNA polymerase results in transcription arrest and conversion of TOP1 cleavable complexes into strand breaks.

DSB repair in phage T4 was also investigated using a physical assay that involved a plasmid substrate with two inverted repeat DNA segments. A DSB introduced into one repeat during a T4 infection induces efficient DSB repair using the second repeat as a template. This reaction was coupled to plasmid replication, was frequently associated with exchange of flanking DNA, and had an absolute requirement for the products of genes *uvsX, uvsY, 32, 46,* and *47 (26).*

DSB repair was demonstrated in an in vitro phage T7 DNA replication and packaging system as well. It was found to be highly recombinogenic, indicating that a recombinational repair mechanism was involved *(48).*

2.5. Mismatch Repair

When two T4 phage, differing by mutation, infect the same host cell they may recombine with each other, thereby generating a heteroduplex region of DNA as an intermediate in recombination, with base mismatches. Base mismatches can be repaired—that is, converted to standard base pairs—during the recombination process. This repair appears to occur by a pathway that includes sequential action of endonuclease VII (Gp49), the $3' \rightarrow 5'$ exonuclease of the T4 DNA polymerase (Gp43), the DNA polymerizing activity of Gp43, and the DNA ligase (Gp30) (reviewed in *62,63*). In this proposed pathway, endonuclease VII cleaves a strand at a mismatched nucleotide producing a 3′-unpaired end, and T4 DNA polymerase excises the nonmatched single strand (with its $3' \rightarrow 5'$ exonuclease) and then fills in the resulting gap. Two mutants (defective in different genes) lacking mismatch repair have been isolated *(30).*

2.6. Post-Replication Recombinational Repair

The concept of post-replication recombinational repair (PRRR) was first proposed by Harm in 1964 for phage T4 and, at about the same time, by Howard Flanders for *E. coli* (reviewed in *6*). In phage, PRRR is a repair process that occurs in single infections (in contrast to MR). In phage T4, mutants defective in PRRR are identified by their (1) increased sensitivity to DNA-damaging agents in single infections compared to wild-type, and (2) reduced genetic recombination. By these criteria, the products of genes *32,46,47,59, uvsW, uvsX,* and *uvsY* are considered to be involved in PRRR (reviewed in *4,6*). A pathway for PRRR in phage T4 was proposed on the basis of the functions of the gene products that appear to be involved in the process, using the better understood PRRR in *E. coli* as a model *(6).*

2.7. Luria-Latarjet Effect

As reviewed by Hyman *(33),* Luria and Latarjet in 1947, using phage T2, carried out one of the earliest studies of DNA repair, although the result they described, later designated the Luria-Latarjet effect, was not interpreted in terms of DNA repair at the time of the study. The Luria-Latarjet effect is an increase in resistance of a virus to treatment by a DNA-damaging agent during the course of infection of host cells. Although first demonstrated in phage T2 using UV as the DNA-damaging agent, the Luria-Latarjet effect was later demonstrated with phages T4 and T5, and could be observed with X-rays or ^{32}P as the damaging agent (reviewed in *33*). Evidence was obtained, using a variety of phage DNA repair defective mutants, that the Luria-Latarjet effect is owing to three repair pathways; excision repair, PRRR, and MR *(33).* The Luria-Latarjet effect appears to develop in two stages. The first stage starts soon after infection and involves excision repair or PRRR. The second stage appears to begin after the first round of DNA replication is complete. DNA damage occurring at this stage can apparently be repaired by MR as well as the other two repair pathways.

2.8. Error-Prone Repair

In phage T4, the hypothesis that mutagenesis induced by UV and other DNA damaging agents occurs by error-prone repair is an attractive explanation for a number of observations (*see 4,6* for review of early work). A mutation in gene *30* (DNA ligase)

decreased UV and HNO_2 mutagenesis, and anti-mutator alleles of gene *43* (DNA polymerase) were found to reduce UV and psoralen-plus-UVA-light (PUVA) mutagenesis (reviewed in *4; see* also *22*). Mutations in other components of the replicative complex, i.e., Gps 32, 41, 44, and 45, increased UV-induced mutagenesis (reviewed in *4*). Mutagenesis induction by UV and ionizing radiation during single infections proceeds via a pathway that depends on UvsW, UvsX, and UvsY *(18).* Because these three gene products are necessary for recombinational repair, it appears that the DNA synthesis step(s) of a recombinational repair process (possibly PRRR) may be less accurate, or more inclined to trans-lesion synthesis, than the DNA synthesis associated with chromosome replication. In contrast, MR of UV-damaged phage appears to be an accurate process *(74).*

2.9. Replication Repair

As reviewed by Kreuzer and Drake *(43),* replication repair was initially identified by the finding of phage T4 mutants with increased sensitivity to MMS in a genetic background that was defective in recombinational repair. Later, mutants with increased UV sensitivity were found in a genetic background defective in both base-excision repair and recombinational repair. These mutations, which occurred in genes *32* or *41,* were thought to define a new repair pathway. Replication repair has not yet been identified in other organisms than phage T4, and its mechanism remains unknown.

3. ENZYMES EMPLOYED IN DNA REPAIR AND THEIR MECHANISMS OF ACTION

3.1. DenV of Phage T4

DNA endonuclease V, or DenV, is a small protein of 137 amino acids with N-glycosylase/β-lyase activity (reviewed in *44*). Its three-dimensional structure has been determined by X-ray crystallography *(51).* The enzyme scans nontarget DNA sequences by electrostatic interactions to search for damaged sites and, subsequently, specifically recognizes the pyrimidine dimer site. As can be seen from Table 1, the presence or absence of DenV does not alter susceptibility to lethal damage caused by agents other than UV light. The basic concave surface of endonuclease V interacts with double-stranded DNA that is sharply kinked at the pyrimidine dimer. There appear to be several specific interactions with phosphate groups on both strands occurring in the minor groove of the DNA. DenV is thought to act by flipping an adenine complementary to one of the thymines in the dimer into a cavity on the protein surface. The flipping may allow the enzyme to discriminate between damaged and normal DNA, and to generate an empty space within the DNA helix, to which catalytically important residues in the enzyme can gain access. Several of the specific amino acids of DenV essential for enzymatic activity have been identified (reviewed in *67,70*).

DenV appears to act by a mechanism common to that of other N-glycosylases that have an AP lyase activity such as *E. coli* endonuclease III, and formamidopyrimidine DNA glycosylase. All three enzymes have similar specificities in recognizing, and using as substrates, duplex oligonucleotides containing the base-lesion analogs O-methylhydroxylamine- and O-benzylhydroxylamine-modified abasic (AP) sites *(56).*

Table 1
Relative Numbers of Lethal Lesions Produced During Single Infections When Phage T4 Mutants are Defective in the Indicated Genes[a]

		Relative number of lethal lesions[b] produced by						
Gene defect	Function	UV	PUVA	MNNG	MMS	MMC	HNO_2	H_2O_2[c]
None		1.0	1.0	1.0	1.0	1.0	1.0	1.0
30	Ligase	1.2	1.2		2.9		1.0	
32	Single strand binding	1.4		2.6		1.1	1.3	0.7
41	Helicase	1.2			2.1			
43	Polymerase	1.1	1.2					
46	Exonuclease	1.4	1.5	3.0		1.4	1.2	0.6
47	Exonuclease	1.2	1.5	3.4		1.7	1.3	0.8
58–61	Primase	1.8						
59	Loads 41 helicase	1.6			2.8			
denV	Endonuclease	2.2	1.0	1.0	1.0	1.0	1.0	1.0
uvsX	Strand transfer	1.7	1.6	1.9	1.8	1.5	1.2	0.8
uvsY	Stabilize UvsX	1.7	1.6	1.6	1.9	1.5	1.2	
uvsW	Helicase	1.5	1.4		1.8			
39[d]	Topoisomerase		0.6			1.2		
52[d]	Topoisomerase		0.4			1.3		
60[d]	Topoisomerase	0.5			1.2			
rnh[e]	RNase, exonuclease	1.5						

[a] The values in the table were taken as the largest values reported in reviews by Bernstein *(4)* and Bernstein and *W*allace *(6),* except as indicated by superscripts.

[b] When applied to free phage or to phage-host complexes, all agents caused inactivation of the ability to produce infective centers. In general, when the log of the surviving fraction was plotted vs the dose of the agent, the survival curve was a straight line, although in the case of some UV irradiation curves there was a very small initial shoulder; that is, except for these small shoulders, survival curves represented killing with single-hit kinetics, following the equation $N/N_0 = e^{-kd}$, where N_0 is the number of phage present in an initially chosen population, N is the number surviving phage after treatment, d is the dose of the inactivating agent, and k is the number of lethal lesions introduced per unit dose. The numbers in the table represent $k_{mutant}/k_{wild\text{-}type}$.

[c] These values are from Chen and Bernstein *(14).*

[d] These values are calculated from data presented in Miskimins et al. *(50).*

[e] This value is from Woodworth and Kreuzer *(72).*

3.2. UvsX of Phage T4

The UvsX protein, in association with the products of genes *uvsY, dda,* and *32,* catalyzes homologous DNA pairing and an efficient in vitro strand-transfer reaction *(38,39,40,77,78).* These reactions are thought to be a central feature of any recombinational repair process. As discussed later (Subheading 7.2.), UvsX is a homolog of *E. coli* RecA *(23).* UvsX, like RecA, can assimilate linear single-stranded DNA into homologous superhelical duplexes to produce D-loops. A necessary prerequisite for UvsX protein-mediated pairing is the polymerization of this protein along the invading single strand, a process known as presynapsis. UvsY and the product of gene *32* are involved in this process as well *(34,38).* UvsX, UvsY, and Gp32 are all cooperative single-stranded DNA-binding proteins. UvsY binds specifically to both Gp32 and UvsX. These contacts allow UvsY to mediate binding of UvsX to Gp32-covered single-

stranded DNA. UvsY also acts to stabilize the filament. A series of events involving multiple protein-DNA and protein-protein interactions is required to mediate a transition from an initial Gp32-DNA complex to a mature presynaptic filament in which the UvsX and UvsY proteins are in contact with the DNA and each other, while most or all of the Gp32 is removed from the complex (reviewed in *34*).

The efficiency of UvsX protein-mediated joint molecule formation between supercoiled duplex DNA and oligonucleotides has a sharp dependence on the degree of homology *(60).* The reaction proceeds efficiently with oligonucleotides containing 32 homologous positions but not with oligonucleotides containing only 24 homologous bases. Even a single base-pair mismatch in the middle of a region of 40 homologous nucleotides had a detectable negative effect on the efficiency of pairing.

Mutants defective in the UvsX protein have increased sensitivity in single infections to inactivation by many agents including UV, PUVA, N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), MMS, MMC, HNO_2, and H_2O_2 (*see* Table 1), suggesting that UvsX is necessary for a recombinational repair process that can occur in single infections, presumably PRRR (reviewed in *4,6*). Mutants defective in *uvsX* are also deficient in MR in response to damage caused by UV, HNO_2, and H_2O_2 (*14;* also reviewed in *4,6*).

3.3. UvsY of Phage T4

The UvsY protein is required for efficient recombination in T4 infected *E. coli.* It is an accessory protein for catalysis of strand exchange *(28).* UvsY stimulates the homologous pairing catalyzed by UvsX. UvsY accelerates loading of UvsX onto Gp32-covered DNA and stabilizes UvsX single-stranded DNA complexes (reviewed in *59*). The mechanism of filament stabilization seems to involve a slower loss of UvsX subunits. These presynaptic filaments are one of the early essential intermediates in the strand-exchange reaction between homologous single- and double-stranded DNAs. In general, mutants defective in *uvsY* are similar to mutants defective in *uvsX* in their pattern of increased sensitivity in single infection to a variety of DNA damaging agents (Table 1), and in their reduced ability to carry out MR (reviewed in *4,6*).

3.4. Gene Product 32 of Phage T4

T4 Gp32 protein is a helix-destabilizing protein that stimulates UvsX protein catalyzed synapsis *(78)* (*see* Subheading 3.2.). Gp32 appears to have a role in both the formation of joint molecules and in Gp41 helicase-catalyzed polar branch migration (*41; see* also Subheading 3.5.). Phage mutants defective in gene *32* have decreased recombination and increased sensitivity to various DNA damaging agents upon single infection (Table 1), implying that Gp32 is necessary for PRRR (reviewed in *4,6*). These mutants are also defective in MR of phage damaged by UV, HNO_2, MMC, and H_2O_2 *(14;* also reviewed in *4,6).*

Gp32 may also have a role in base-excision repair *(53).* This protein has a strong affinity for nicked AP DNA, suggesting that it may displace DenV from a damaged site once the enzyme has completed its enzymatic reaction, thus allowing DNA polymerase to fill the gap *(65).* The binding of Gp32 to nicked AP sites could also prevent resealing of the DNA by ligase before excision of the damage.

3.5. Gene Product 41 of Phage T4

The Gp41 helicase exhibits a single-stranded DNA-stimulated GTPase/ATPase activity and a 5′ to 3′ DNA helicase activity that is driven by ATP/GTP hydrolysis. Gp41 translocation is processive (and uni-directional) along DNA (reviewed in *21*). Although UvsY is required for homologous pairing, it strongly inhibits branch migration catalyzed by the UvsX protein. Polar branch migration is completely dependent on the gene *41* helicase. The helicase is delivered to the strand-exchange complex by the gene *59* accessory protein in a strand-specific fashion through direct interactions between Gp59 and Gp32 *(59).* The Gp41 helicase functions in both replication and recombination. However, a gene *41* mutation was isolated that specifically impairs recombination and not DNA replication, implying that the recombination function of Gp41 is at least partially separable from the replication function *(76).* Gene *41* mutants are deficient in MR of UV-damaged phage and have increased sensitivity to both UV and MMS in single infections, implying a defect in PRRR (reviewed in *4,6*) (also *see* Table 1).

3.6. Gene Product 59 of Phage T4

Phage T4 gene *59* encodes a protein that facilitates the loading of the T4 helicase (Gp 41) onto recombinational intermediates *(1,52).* Mutants defective in gene *59* have increased sensitivity to UV, MMS and X-rays in single infections, and also have reduced ability to carry out MR of UV-induced damages (reviewed in *4,6*).

3.7. Gene Products 46 and 47 of Phage T4

The products of genes *46* and *47* apparently form a recombinational exonuclease *(49),* but the enzyme has not been purified nor studied extensively. Gene *46* and *47* mutants are defective in both MR and PRRR subsequent to treatment with a wide range of agents (UV, HNO_2, MMC, ethyl methanesulfonate, MNNG, and H_2O_2) (*14;* also reviewed in *4,6*) (also *see* Table 1). Possible homologs of genes *46* and *47* have been found in phage T5 *(10).*

3.8. Junction Resolving Enzymes of Phages T4, T7, and T3

The latter stages of general recombination processes, including recombinational repair, are thought to require the resolution of four-way DNA junctions, also referred to as Holliday structures. This involves enzymes that can both recognize and manipulate these DNA structures. Such enzymes, called X-solvases or resolvases, have been isolated from a wide variety of sources including phage, eubacteria, yeast, mammals, and their viruses (reviewed in *27,36,71*).

Phage T4 endonuclease VII (Gp49), a 157 amino acid protein, is a well-studied example of this class of enzymes (reviewed in *36*). It cleaves four-way junctions by introducing symmetrical cuts in the two strands of like polarity. The nicks can subsequently be sealed by DNA ligase. T4 endonuclease VII has a broader range of substrate specificities than most X-solvases and, besides four-way junctions, it can also cleave three-way junctions, single-strand overhangs, nicks, gaps, heteroduplex loops, base mismatches, curved DNA, and bulky adducts (reviewed in *9*). Endonuclease VII has several domains *(27).* Towards the N-terminal end of the protein lies a section of polypeptide in which four cysteine residues, distributed in a CxxC—CxxC pattern, coordinate one atom of zinc; and in the C-terminal region lies a 31 amino acid sequence

that is 48% identical with a sequence found in the DNA repair protein DenV (*see* Subheading 3.1.). This sequence of endonuclease VII can be replaced with the corresponding sequence from T4 DenV with no change in the pattern of cleavage of four-way junctions *(27).* A dimerization domain has also been identified *(9).*

In phage T4 infections, another four-way resolvase may be present that can partially compensate for loss of endonuclease VII. Mutations in gene *49* were found to reduce genetic recombination, but not MR *(32),* and repair of DSBs was only partially reduced by gene *49* mutations *(26).* Endonuclease VII appears to play a key role in mismatch repair (*see* Subheading 2.5. above) and may therefore contribute to gene conversion, which is thought to occur by heteroduplex repair during genetic recombination *(63).*

Phage T7 undergoes genetic recombination during infection, and this is reduced by mutants defective in T7 endonuclease I (Gp3) (reviewed in *36,71*). This enzyme has been shown to cleave branched DNA species including four-way junctions. The junction-resolving enzymes from T7 and T4 are functionally quite similar. At the amino acid-sequence level, however, there is little similarity between the two, except for a region of about 45 bases where they share 32% identity. Phage T3 encodes an enzyme that is required for genetic recombination and is very similar in sequence and function to T7 endonuclease I.

3.9. The dda Gene of Phage T4

The T4 Dda protein is a DNA helicase that is required to move the T4 replication complex past DNA template-bound proteins in vitro. This helicase also allows the phage-recombination machinery to drive the branch-migration reaction, which the UvsX protein catalyzes, through a RNA polymerase promoter complex *(39,58).* Mutants defective in *dda* are not grossly radiation-sensitive and recombination-deficient, suggesting that another helicase may substitute for the Dda protein, if necessary. (It may be speculated that Gp41 helicase complexed with Gp59 might provide this function.) *(58).*

3.10. UvsW of Phage T4

Mutants of phage T4 defective in *uvsW* have reduced spontaneous recombination and increased sensitivity to UV, PUVA, and MMS (Table 1). UvsW is necessary for normal levels of both MR and PRRR (reviewed in *4,6*). Analysis of the *uvsW* gene *(20),* indicates that the promoter region contains a sequence resembling the consensus for T4 late promoters, and that *uvsW* is expressed as a late gene. UvsW appears to be a helicase that catalyzes branch migration and dissociation of RNA-DNA hybrids *(12).* UvsW was suggested to be the key regulatory factor in the switch from early to late DNA replication *(20).*

3.11. Ligase

Mutants defective in DNA ligase (Gp30) have increased sensitivity to UV and MMS in single-infections (reviewed in *4,6*). The final step in excision repair and recombinational repair is thought to involve the sealing of a phosphodiester bond by DNA ligase. The mechanism of action of DNA ligase involves covalent modification of the enzyme by adenylation, transfer of the AMP residue in a phosphoanhydride linkage to the 5′-phosphate of nicked DNA, and then resealing of the DNA strand using the energy of

AMP hydrolysis. In the absence of ATP, T4 DNA ligase can also act as an AP lyase to catalyze a β-elimination reaction that leads to the removal of an abasic 5′-dRP residue *(11).* The first step in base-excision repair of a pyrimidine dimer in phage T4 infection is the generation of an AP site by the glycosylase activity of DenV, (*see* Subheading 2.1.). The combined action of the AP endonuclease activity of DenV (on the 3′ side of the AP site) and the AP lyase activity of DNA ligase (on the 5′ side of the AP site) removes the AP site leaving a one-nucleotide gap.

3.12. The Gene 2.5 Single-Strand Binding Protein and the Gene 4 Helicase of Phage T7

The gene *4* helicase of phage T7, like the gene *41* helicase of phage T4, mediates DNA-strand transfer between homologous DNA molecules. In phage T7 infected *E. coli,* the gene *4* helicase-mediated strand-exchange reaction is thought to provide the major pathway for recombinational repair *(41).* In phage T7-infected cells, recombinational repair of DSBs is very efficient (reviewed in *42*). The gene *4* helicase acts together with the gene *2.5* single-stranded binding protein to promote the annealing of homologous regions of two DNA partners to form a joint molecule and then strand transfer. In this reaction T7 Gp2.5 is essential for the formation of a joint molecule, but it is not required for the further T7 Gp4-mediated strand transfer *(41).* T7 gene *4* helicase alone is able to mediate strand transfer, provided that a joint molecule is available. Strand transfer can proceed at a normal rate even if thymine dimers are present in both partners *(42).* Short nonhomologous inserts in either partner can also be tolerated.

4. MUTAGENESIS AS A SIDE-EFFECT OF REPAIR

As pointed out by Holmquist *(29),* a major function of base-excision repair is to minimize mutation resulting from endogenous lesions. However, glycosylases employed in base-excision repair, such as DenV, are not completely specific for damaged sites, but at some frequency they remove normal bases, producing AP sites unnecessarily. There is an error frequency for repairing AP sites, which generates spontaneous mutations. As Holmquist notes, the mutation rate in phages T2 and T4 is maintained by evolutionary pressure to be at about the same rate as in other haploid microorganisms, i.e., about 0.003 mutations per genome per replication. This implies selective pressure to maintain DenV at a balanced level that is sufficient for repair but not so high as to generate excessive mutations.

5. EFFECTIVENESS OF DIFFERENT REPAIR MODES

In Table 1, we summarize rates of lethal hits delivered to mutants relative to lethal hits delivered to wild-type phage T4 during single infections. The largest relative inactivation rate in Table 1 is 3.4 for a gene *47* mutant treated with MNNG. It can then be calculated that when the gene *47* product is functional, (3.4–1.0)/3.4, or 71% of the lethal MNNG lesions present during a mutant infection are repaired by the gene *47* product in a wild-type infection. This calculation may underestimate the fraction of lethal lesions that can be repaired in a single infection, since other repair pathways, not involving gene *47* function, might remove additional lesions caused by this agent. In the cases of UV and MMS damage there are three pathways of repair that operate during a single infection (reviewed in *6*). In these cases we can use $K_{\text{triple-mutant}}/K_{\text{wild-type}}$ values of

Table 2
Measured Levels of Repair During Single and Multiple Infections[a]

	Singe infections	Multiple infections	
Agent	Percent lesions repaired[b]	MR factor	Shoulder repair
UV	82	4.2	18
MNNG	71	2.9	0
MMS	64	—	—
MMC	41	36.0	0
X rays	41	4.0	27
PUVA	—	11.0	0
HNO_2	23	5.0	0
^{32}P	—	4.6	0
H_2O_2	—	8.3	2

[a] Reviewed in *(6, 14).*

[b] These values were calculated using $k_{mutant}/k_{wild\text{-}type}$ values from Table 1 except for UV and MMS. For UV and MMS the calculations are described in the text.

mutants defective in all three pathways. These triple mutant values are 5.5 for UV and 2.8 for MMS. In Table 2, we give the calculated percentages of lethal lesions known to be repaired in single infections for the agents listed, using values from multiple pathways where known.

Data from MR experiments are presented by plotting log of surviving infective-center-forming ability of multiply infected cells versus dose of an inactivating agent. These MR survival curves are usually declining straight lines, or declining straight lines with an initial shoulder. Over the declining straight line region of the curve, the inactivation kinetics can be represented in a form similar to the inactivation kinetics of singly infected cells, as $N/N_0 = e^{-kd}$, where d is the dose of agent applied and k is the inactivation constant, indicating the lethal hits delivered per unit dose of agent. To compare the survival of singly infected cells and multiply infected cells, an MR factor can be calculated. The MR factor is given by k_{mono}/k_{multi}, where the k values are taken from the straight line declining portions of the curves. Figure 1 shows a hypothetical set of curves where the MR factor is 4.0. This hypothetical set of curves also shows a hypothetical single infection by mutant phage where the relative number of lethal lesions delivered to the mutant infected cells, compared to wild-type infected cells, is 2.0. A shoulder is shown in the survival curve representing the multiply-infected cells (designated wt MR). The length of the shoulder is defined by the dose of inactivating agent needed to reach the transition to the straight line declining portion of the curve, and in this figure the shoulder is set at 4.0. Such shoulders, which occur in MR curves obtained with a number of agents, appear to reflect a type of saturable recombinational repair. As reviewed in Chen and Bernstein *(14),* for most agents, the shoulder on an MR curve is removed in the presence of mutations causing defects in recombination. Together, the MR factor and the shoulder repair indicate the additional repair available in multicomplexes compared with monocomplexes.

Table 2 lists the MR factors and shoulder repair values that have been found for several agents. MR is a very powerful form of repair. For agents that give rise to large MR factors or large shoulder-repair factors, such as UV, MMC, X rays, and H_2O_2, (similar

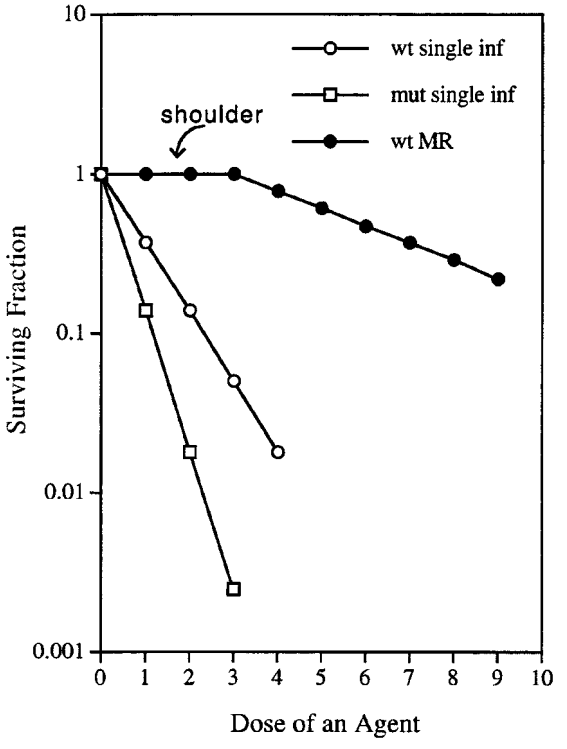


Fig. 1. Hypothetical inactivation curves to illustrate relative survival of infected cells where the infecting phage are: wild-type, infecting cells singly (open circles, designated wt single inf); mutant, infecting cells singly (open squares, designated mut single inf); or wild-type, infecting cells with a multiplicity greater than or equal to 2.0 (filled circles, designated wt MR). Shoulders are often found on wild-type MR curves, as is indicated here.

to the hypothetical agent in Fig. 1), cells singly infected by wild-type phage and treated by the agent may give survival of less than 0.1% when multiply infected cells give survival near 100%.

6. COMPLEMENTATION OF REPAIR DEFECTS IN OTHER SPECIES BY *DENV*

The phage T4 *denV* gene, which codes for endonuclease V, has been introduced into *E. coli, Saccharomyces cerevisiae,* mouse and human cells. In *E. coli, denV* complements nucleotide excision-repair mutants defective in *uvrA, uvrB, uvrC,* and *uvrD* as well as recombination-repair mutants defective in *recA, recB,* and *recC (69).*

In immortalized repair-proficient mammalian cells, endonuclease V activity significantly increases the rate and overall extent of pyrimidine dimer removal *(37,46).* When murine epithelial cells were transfected with the *denV* gene, pyrimidine dimer repair was enhanced two-to threefold *(45).* In control cells, not transfected with *denV,* the patch size for excision repair of DNA photoproducts was estimated to be 34 nucleotides

per photoproduct removed. However in the *denV*-transfected cells, a smaller average patch size of 10–16 nucleotides per photoproduct removed was found *(45).* Thus, endonuclease V activity appears to alter not only the extent, but also the nature of excision repair in UV-exposed mammalian epithelial cells. In humans, *denV* partially complemented the nucleotide excision-repair defect in xeroderma pigmentosum mutant cells (complementation groups A, C, and E) in terms of restoration of colony-forming ability and excision-repair synthesis after UV irradiation *(17,68).* DenV, when topically applied within liposomes to the skin of XP patients, allowed fewer cyclobutylpyrimidine dimers to accumulate in DNA, and reduced erythema *(75).* Thus DenV appears to have therapeutic potential for XP patients.

Cockayne syndrome (CS) is an autosomol recessive disorder characterized by hypersensitivity to UV light and a defect in the preferential repair of UV-induced lesions in transcriptionally active DNA by the nucleotide excision-repair pathway. Expression of *denV* in CS (complementation group A) cells resulted in partial correction of the UV-sensitive phenotype in assays of gene-specific repair and cell viability, while correction of CS (complementation group B) cells in the same assays was minimal or nonexistant *(24).* As described earlier, DenV is a glycosylase that is specific for cyclobutane-pyrimidine dimers, and DenV-incised lesions are believed to be processed via the base excision-repair pathway. The inability of DenV to complement the nucleotide excision-repair defect in CS-B cells to normal levels, suggests that the CS-B gene may have a role in base-excision repair *(24).*

Transgenic tobacco plants expressing phage T4 *denV* exhibited varing degrees of increased, rather than decreased, sensitivity to UV-C light and the alkylating agent dimethyl sulfate *(47).* This suggests that a defect arises when the plants try to repair AP sites. AP sites should be introduced into UV-irradiated DNA by the DenV glycosylase, and into alkylated DNA by the spontaneous elimination of alkyl-purines. They are the only lesions generated by both agents. These may be further cleaved by the AP lyase activity of DenV, but in a manner that produces a cleavage product poorly adapted to pre-existing tobacco base-excision repair pathways *(47).*

7. HOMOLOGIES OF PHAGE DNA REPAIR PROTEINS WITH PROTEINS OF BACTERIA AND EUKARYOTES

7.1. DenV of Phage T4

As reviewed in Furuta et al. *(25)* and Krokan et al. *(44),* DenV homologs have been found in *E. coli, Micrococcus luteus,* and *S. cerevisiae,* although the sizes and amino acid sequences of the enzymes from these organisms differ substantially from those of DenV. Recently, however, a fairly close homolog of DenV was found in the virus PBCV-1. This homolog has 41% amino acid identity to DenV *(25).* The virus PBCV-1 replicates in certain eukaryotic chlorella-like green algae present as endosymbionts in some isolates of *Paramecium bursaria.* Furuta et al. *(25),* using a probe that hybridizes to the *denV* homolog in PBCV-1, assessed 42 other viruses of chlorella, and found that their probe hybridized strongly to the DNA of 37 chlorella viruses and weakly to the other five virus DNAs. The *denV* homolog from PBCV-1 was also able to complement the DNA damage-repair defect of an *E. coli* mutant defective in *uvrA* and *recA*.

7.2. Gene Product UvsX of Phage T4

The *E. coli* RecA protein is unable to complement mutants defective in UvsX *(77).* Nevertheless, analysis of the primary sequence relationships of UvsX to the three-dimensional structure of RecA from *E. coli* suggests that UvsX is a structural homolog of the bacterial RecA protein *(64).* By similar criteria, the Dmc1 protein from *S. cerevisiae* also appears to be a structural homolog of RecA *(64).* These analyses argue that proteins in this group are members of a single family that diverged from a common ancestor that existed prior to the divergence of prokaryotes and eukaryotes. Dmc1 has a meiosis-specific function required for meiotic recombination, and thus the study of UvsX function may illuminate aspects of meiosis.

7.3. Gene Products 46 and 47 of Phage T4

The *rad52* gene of *S. cerevisiae,* necessary for recombinational repair, was found to complement phage T4 mutants defective in genes 46 and 47, implying similarity of function of the yeast and phage gene products *(15).*

7.4. Ligases of Phage

DNA ligases fall into two broad classes, those that are NAD-dependent and those that are ATP-dependent. These two classes differ in the source of the AMP molecule that becomes linked to the ε amino group of lysine in the ligase, as the first step in the ligation reaction. Whereas the NAD-dependent ligases are found exclusively in eubacteria, the ATP-dependent ligases are found ubiquitously in eukaryotes and archaea *(16).* The ATP-dependent DNA ligases are also found in the T-even phages T4 and T6, the T-odd phages T3 and T7, in eukaryotic DNA viruses and, recently, in *Haemophilus influenzae (16).*

8. RECOGNITION OF DAMAGED SITES IN DNA

8.1. DenV

The co-crystal structure of T4 endonuclease V in complex with a DNA duplex containing a thymine dimer has been reported *(70).* The three-dimensional structure of the complex refined at 2.75 angstrom resolution revealed the unique structure of the bound DNA duplex. Contrary to what one might expect, there was no interaction of the enzyme with the cross-linked moiety of the pyrimidine rings. On the other hand, there was extensive interaction between the amino acid side-chains of DenV and the deformed phosphate backbones in the vicinity of the pyrimidine dimer.

8.2. Gp32

Gp32 binds with high specificity to single-stranded DNA. It also binds more efficiently to double-stranded DNA modified either with cis-diaminodichloroplatinum(II) or with aminofluorene derivatives than to native DNA *(65).* This increased affinity is related to the formation of locally unpaired regions, which are strong binding sites for the single-strand binding protein. On the other hand, Gp32 has the same low affinity for native DNA and DNA containing methylated purines and other types of damage that do not induce sufficient structural change to allow Gp32 binding. Despite its cooperative mode of binding to single-stranded DNA, Gp32 alone is not able to melt damaged DNA. Therefore only a limited number of protein monomers bind to each damaged site *(66).*

8.3. DNA Polymerase of Phage T4

Damaged sites in the template strand of DNA may be recognized by blockage of the movement of DNA polymerase during replication. DNA adducts of the environmental carcinogen benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE) interact stereospecifically with prokaryotic and eukaryotic polymerases in vitro. Of six different DNA polymerases tested for their ability to replicate past different diastereomers of BPDE at specific sites in the DNA, the T4 DNA polymerase (Gp43) was most strongly inhibited *(13).* However T4 DNA polymerase can replicate past O^6-methylguanine residues in the presence of Gp45 accessory protein that clamps the polymerase to the DNA template *(57).*

9. CONCLUDING REMARKS: ADAPTIVE CONSEQUENCES OF DNA REPAIR

We have reviewed the main processes of DNA repair encoded by phage genomes. Phage T4 was emphasized because most of the experimental work on DNA repair in phage was performed with this organism. The repair processes in phage T4 are of two kinds. The first kind, including DenV mediated base-excision repair and photoreactivation, is specific for one type of damage, pyrimidine dimers. The second kind, including MR, double-strand break repair and PRRR, are recombinational repair processes. In principle, it is only when two or more genomes (or portions thereof) are present that the important class of double-strand damage can be accurately repaired. As reviewed by Bernstein and Wallace *(6),* these recombinational repair processes appear to reflect a common underlying mechanism mediated by a common set of gene products that can repair a wide variety of DNA damages. As described in Subheading 5, MR (or the sexual mating process of multiplicity reactivation) is a particularly powerful form of recombinational repair.

UvsX, a RecA/Dmc1 homolog, plays a central role in all of the recombinational repair processes. The function of recombination during meiosis in eucaryotes and analogous processes in procaryotes is currently a hotly debated issue in the context of the adaptive advantage of sex, a major unsolved problem in biology. There are two opposing views on this issue. The first is that recombination, and hence sex, is primarily an adaptation for promoting allelic variation (reviewed in *2*). The second view is that recombination is primarily an adaptation for DNA repair and that allelic variation is a byproduct (reviewed in *7,8*). Cox *(19)* has argued, from an enzymological perspective, that the RecA protein evolved as the central component of a recombinational DNA repair system, with the generation of genetic diversity as a sometimes beneficial byproduct. One of the arguments presented by Cox is that RecA protein binding, which initiates recombination, is targeted to perturbed, or underwound regions of duplex DNA that can result when DNA is damaged, a characteristic expected if recombination is primarily an adaptation for repair. In phage T4, the similar UvsX recombination protein depends on Gp32, which (*see* Subheading 8.2.) is targeted to DNA damage. An experiment was performed using multiple infection in phage T4 as a model of sexual interaction, to test the concept that the adaptive advantage of sex is promotion of recombinational repair *(5).* When an undamage phage T4 injects its DNA into a host cell, it establishes a barrier to infection by a second phage T4 within 2 min. This promotes asexual reproduction and the preservation of the host cell as a resource solely for the first phage. However if the first phage is

treated with UV, its barrier to superinfection is reduced. Thus, damage to a first-infecting phage shifts reproduction from an asexual mode towards a sexual mode, as would be expected if mating is an adaptation for repair. Furthermore, genes from the damaged first-infecting phage were shown to have a much enhanced survival owing to the shift towards sexual reproduction. Overall in phage T4, there is strong experimental support for the concept that recombination is primarily an adaptation for DNA repair, a process vital for immediate survival, and little, if any, evidence that the generation of recombinational variation is of substantial benefit.

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2

Post-Replication Repair

A New Perspective Focusing on the Coordination Between Recombination and DNA Replication

Steven J. Sandler

1. INTRODUCTION

The repair of DNA is crucial to the survival of every organism. Organisms have evolved many biochemical pathways for detecting and repairing DNA damage with high fidelity. Failure to repair DNA with high fidelity leads to a high mutation frequency. This in turn is correlated with a high risk of cancer in humans.

The type of DNA damage usually dictates the type of DNA repair pathway used by the cell. This article focuses on post-replication repair (PRR) of DNA. This process was first noted while using ultraviolet (UV)-irradiation as a source of DNA damage. While there are several types of UV-induced lesions (i.e., pyrimidine dimers), most are removed by the nucleotide-excision repair (NER) and photoreactivation repair (PR) pathways. However, when a replication fork encounters one of these noncoding lesions (not removed by NER or PR), a special type of recombinational repair pathway is available to repair the DNA damage. This type of repair has been referred to both daughter-strand gap repair (DSGR) and PRR. In recent years PRR has become synonymous with the RecF pathway of recombination in *Escherichia coli.* The RecF pathway of recombinational DNA repair operates on gapped DNA substrates that presumably arise after the replication fork has partially replicated past a noncoding lesion in the template DNA (leaving a gap opposite the noncoding lesion). The missing information (forming the gap) across from the noncoding lesion is then supplied by the other daughter duplex DNA in a process requiring RecA. In this process, information from the fully replicated daughter, complementary to the lesion, is placed across from the lesion. Thus the damage is now in a DNA duplex and can be repaired either NER or PR.

Recently PRR has been integrated into a broader paradigm that explains the coordination of DNA repair, DNA replication, and recombination in the cell. This paradigm, called CPR (*c*oordinated *p*rocessing of damaged *r*eplication forks) *(19,20,80),* combines many other aspects of DNA metabolism and cell division. CPR emphasizes that these processes are used by the cell in a housekeeping sense (without the occurrence of

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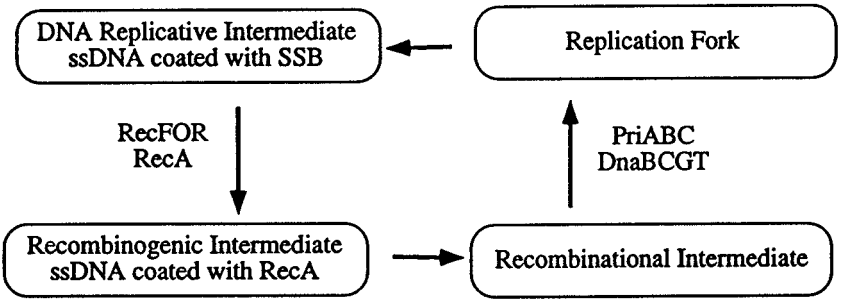


Fig. 1. Proposed relationship between DNA replication and recombination intermediates. The gene names along the vertical arrows indicate the proteins that perform multiple steps in the conversion between substrates. The reactions between boxes separated by the horizontal arrows are less well-defined. Once again they indicate conversion between one recombination or replication structure and another.

extra DNA damage). In a simple sense, CPR is the process by which collapsed or arrested replication forks are repaired by recombinational processes and then restarted. It is thought that restart is an essential process. It is important to emphasize that some of these repair and restart processes may be *recA*-independent since *recA* mutants are viable. Other aspects of CPR include chromosome partitioning (*dif*locus and *XerCD*) *(91)* and cell division (*ftsK*) *(91).*

Thus this review will explain PRR in terms of CPR and will focus on two groups of proteins thought to catalyze opposite reactions in the cell (Fig. 1). The first group includes the RecF, RecO, and RecR (RecFOR) proteins and are thought to catalyze presynaptic steps in PRR that convert a DNA replicative intermediate, ssDNA coated with SSB to a recombinogenic intermediate, ssDNA coated with RecA *(14,78).* The assembly of a RecA-ssDNA filament is critical for molecular healing of the damaged replication fork by recombination. The second group of *E. coli* proteins were initially characterized as elements required in vitro DNA replication of ΦX174 ssDNA phage. Their role in *E. coli* however, has only recently begun to be appreciated. These proteins, collectively called the primosome assembly proteins, include PriA, PriB, PriC, DnaT, DnaC, DnaB, and DnaG (reviewed in *34,57,58,81*). They are thought to restart DNA replication forks at recombinational intermediates and to be essential for normal vegetative growth. Thus they complete the transition in PRR, allowing a collapsed replication fork that has been repaired by the action of RecFOR, RecA, and the process of homologous recombination to be restarted.

The reader is also referred to other articles that review aspects of PRR *(37,38),* the homologous recombination machinery in general *(14,35,46,48), recFOR* specifically *(72),* CPR *(20,57,80),* and the primosome assembly proteins *(34,57,58,81).*

2. AN OVERVIEW OF HOMOLOGOUS RECOMBINATION

Recombination is often thought of as occurring in three distinct stages. The first stage is called pre-synapsis. In this stage, one of the two interacting duplexes of DNA is tailored so that it can be bound by the RecA protein. This tailoring often involves the generation of ssDNA through the action of helicases that unwind duplex DNA or sin-

gle-stranded exonulceases that selectively degrade one strand of DNA. Once the ssDNA is generated, it can be bound by RecA to create a protein-DNA helical filament. It is this filament that is thought to be the active agent that searches for a homologous region of duplex DNA. How RecA performs this search is still an unsolved aspect of RecA biochemistry. Several models have been proposed to explain RecA strand pairing, recognition of homologous regions, and strand exchange *(8,51,86).* Once a homologous region is found, RecA can catalyze the invasion of the ssDNA into the duplex DNA displacing the identical strand. This DNA structure is often called a displacement loop or D-loop. This structure includes a crossover or Holliday junction *(90)* that can undergo branch migration in which the Holliday junction diffuses along the length of the DNA molecule. The Holliday structures may then be resolved enzymatically in *E. coli* by RuvC or Rus *(87).* These enzymes can cleave two strands of the Holliday structure either vertically or horizontally. The position and way in which these structures are resolved determines the structure and phenotypes of the recombinants. Traditionally, the process of recombination has been thought to end after the post-synaptic steps described. However as will be discussed later, it is now necessary to invoke other post-synaptic steps that include the assembly of a DNA replication fork at a recombination intermediate in order to attain viable recombinants.

2.1. Substrates for Homologous Recombination

When thinking about the molecular process of recombination, it is often instructive to focus on the different types of DNA substrates before introducing the gene products that operate on them. To a large degree, the type of DNA substrate dictates that set of gene products will be needed to perform the recombination event. In this sense recombination is thought to be substrate-limited. The field has largely divided the many different types of DNA substrates into two varieties: duplex molecules with double-strand ends and duplex molecules with regions of ssDNA (gaps). These substrates are operated on by the RecBCD (*see* Chapter 8 in Volume *1*) and RecF pathways of recombination respectively and the latter is described in more detail later. One way to think about these two pathways is that they describe sets of pre-synaptic enzymes that funnel the many different DNA substrates into substrates that can be bound by RecA (Fig. 2). Historically, the RecF pathway genes also included gene products involved in branch migration (*ruvA* and *ruvB*) and Holliday junction resolution *(ruvC).* Now however, these enzyme are thought only to play a post-synaptic role. The roles of *recJ, recN,* and *recQ,* also commonly referred to as RecF pathway genes, in pre- and or post-synapsis are presently unclear.

2.2. Pathways of Homologous Recombination

Conceptually, a recombinational pathway for DNA is very much like a biochemical pathway for the biosynthesis of an amino acid. One has substrates that are acted on by enzymes that produce products. Genetically, if a mutation is introduced that blocks that pathway (when there is only one pathway), a phenotype will be seen. If the cell has multiple pathways that can provide the same end product, a phenotype will only be seen if genes in both pathways are mutated. For recombination, the phenotype seen is often a decrease in recombination frequency. This is often measured by the production of a physically or genetically scoreable recombinant normalized against experimental para-

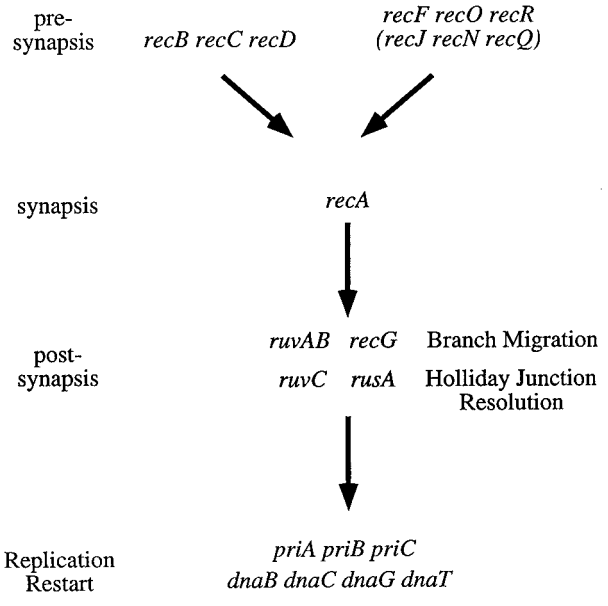


Fig. 2. Genes needed for RecBCD and RecF pathways of recombination in *E. coli.* The genes are grouped to correspond with their different roles in the stages of recombination. The genes in parentheses encode functions whose role is not yet clear. Although the genes involving replication restart have been placed after the post-synaptic steps of branch migration and Holliday junction resolution, this has not yet been demonstrated experimentally. These genes define a minimal set. Other genes know to have an affect on these processes include *ssb, lig,* mismatch repair, and genes encoding the different topoisomerases.

meters. For some years, the use of different recombinational substrates introduced by different protocols (i.e., conjugation, transduction, inter- and intra-plasmidic recombination, and substrates with direct or inverted repeats) caused some confusion. With the recognition that these substrates are physically different and need a different set of recombinases to be processed by the cell, illumination has come to the field.

In *E. coli,* there are commonly thought to be two main pathways for recombination. These have been alluded to earlier and are diagrammed in different ways in Figs. 2 and 3. The major differences between these pathways are in the pre-synaptic steps; the two pathways are named after the genes used in these initial steps. Figure 3 illustrates that the differences in recombination pathways are only in the pre-synaptic steps and that the substrates are processed to common intermediates. Although there is only one synaptic protein listed, the post-synaptic steps of branch migration and Holliday junction resolution require multiple enzymes. The reason for this redundancy is not clear.

The examples in Fig. 3 use replicative DNA substrates in which the DNA damage could result from housekeeping functions or additional insult to the cell. The left side of Fig. 2 shows a standard model for RecBCD mediated double-strand break repair *(88).* In the RecBCD pathway, a duplex DNA with a double-stranded end is produced when the replication fork encounters a nick. In short, the dsDNA end is tailored so that RecA can use it in strand invasion to produce a D-loop. Branch migration and Holliday junction resolution can leave a substrate that is then ready for restarting DNA replication. For additional details, *see* Chapter 8 in Volume 1.

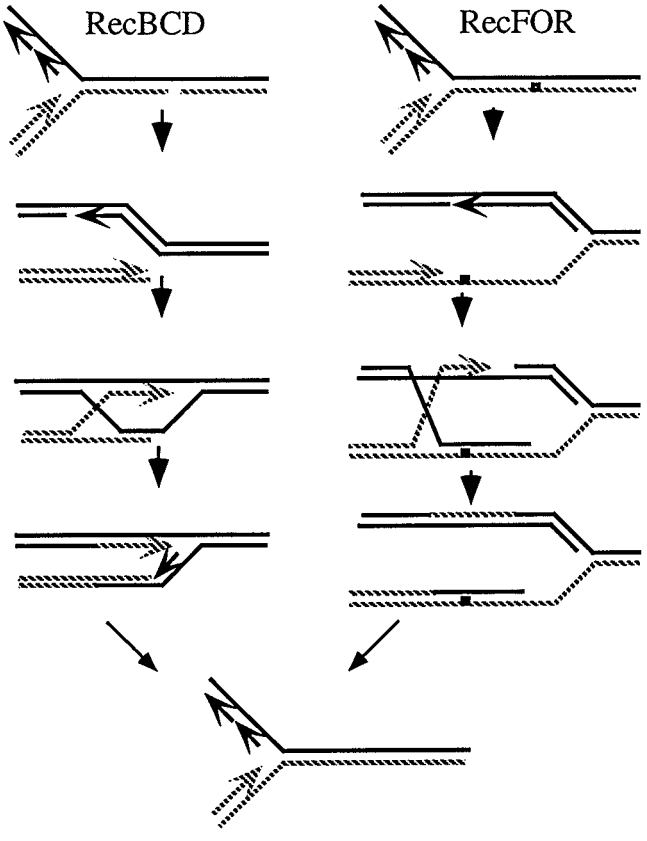


Fig. 3. How the RecBCD and RecFOR pathways of recombination may repair collapsed replication forks and then produce a structure that is suitable for restart of DNA replication. The structures shown include the starting substrate, replication-fork collapse, a synapsis/post-synapsis intermediate, Holliday junction intermediates, a substrate for replication fork restart and the restarted replication fork.

The right side of Fig. 3 shows PRR by the RecFOR pathway of a noncoding lesion. This process conjures up several theoretical problems and questions. How does the replication machinery replicate past the noncoding lesion on only one strand? How far past the lesion does it go? What is the molecular signal for this procedure? What are the required proteins? How large are the gaps produced? Is the process different if the damage is encountered by the lagging or leading strand polymerases? Although clear answers to none of these question are available, the events after production of the proposed gap have been addressed in some detail later.

To complement this broad overview of recombination, PRR and CPR, the remainder of the article will focus on *recF, recO,* and *recR (recFOR)* genes and proteins and then introduce the less well-known and -studied primosome assembly genes.

3. GENETICS OF RecFOR

3.1. Isolation and Characterization of the recFOR Genes

The first mutation found in *recF, recF143,* was identified by its ability to cause extreme recombination deficiency (Rec$^-$ as measured by conjugal recombination) and

UV sensitivity (UV^S) in a *recB recC sbcB sbcC* strain *(28).* Unlike *recA, recB,* and *recC* single mutations that cause both Rec^- and UV^S phenotypes, *recF143* single mutations caused only UV^S. The ability to affect DNA repair and not recombination has been both the hallmark and the riddle of *recF* function in DNA metabolism. Mutations in *recO* and *recR* were subsequently shown to produce virtually identical phenotypes *(26,43,107).* It has been proposed that *recF, recO,* and *recR (recFOR),* act in a biochemical pathway that identifies and converts a DNA replicative intermediate (ssDNA coated with single-stranded DNA binding protein [SSB]) to a recombinogenic one (ssDNA coated with RecA) by helping RecA displace SSB on the proper DNA substrate *(14,78)* (Fig. 1). However, it is still unclear how RecFOR orchestrates the transition from DNA replication to recombination. Research suggests that subcomplexes of RecOR *(85,97),* RecFR *(105,106)* and RecF may also exist in addition to RecFOR *(27)* and may catalyze different parts of the complete reaction or they may have other roles in the cell. RecFOR homologs have only been found in bacteria. Nonetheless, their function appears to be maintained in other evolutionarily diverged systems. For example, yeast genes *RAD52, RAD55,* and *RAD57* have been proposed to have a function analogous to *E. coli* RecFOR *(92,93).*

3.1.1. Phenotypes of recFOR Mutants

Single mutations in the *recFOR* genes cause UV sensitivity *(28,33,55),* attenuation of UV-induction of the SOS response *(26,107),* and decreased plasmid recombination *(15,21,33).* For UV sensitivity, the three genes are epistatic *(13,44,45,47,55).* Other phenotypes of *recF* mutants include defects in mutagenesis of ssDNA phages *(12)* and induction of the adaptive response *(101).* These latter phenotypes have not been tested for *recO* and *recR* mutants. The effect of *recF, recO,* and *recR* mutations in other mutant backgrounds is of great interest. For example, it has been shown in *uvrA (96)* and *priA (73)* mutant strains, that *recF* mutations either decrease the UV resistance or viability of the strain. This argues that *RecF* function is needed in pathways that operate in the absence of these other gene products.

Perhaps the study of *recFOR* has been given less emphasis historically than the RecBCD pathway because these genes were originally perceived to act in an alternate or secondary pathway of recombination in *E. coli.* This is certainly not the case; single mutations in *recFOR* have distinctive phenotypes (e.g., UV^S). The importance of the RecF pathway and its difference from the RecBCD pathway of recombination lies in the types of substrates that it handles. The RecBCD enzyme acts at the ends of linear DNA molecules, whereas the *recFOR* proteins have been associated with recombination in the middle of DNA molecules. Thus, *recB* or *recC* single mutations have major effects on conjugal recombination (linear DNA substrates with ends) and single mutations in *recFOR* affect recombination with plasmid substrates *(15,21,33),* P22 transduction and chromosomal recombination in *Salmonella typhimurium (24,60).* This may not be the only difference from the RecBCD pathway since UV-induced SOS expression by the RecF pathway requires DNA replication *(83,84).* It should also be noted that the RecF pathway is flexible and can be adopted to function on linear DNA substrates during conjugal recombination. Thus in a *recBC sbcBC* strain, *recFOR* mutations have dramatic effects on conjugal recombination *(28).*

Phenotypic studies on the overexpression of *recFOR* genes have revealed interesting and useful information about these proteins. Overexpression of the *recO* protein can

Table 1
Biochemistry of RecFOR proteins and Interactions DNA, SSB, and RecA

	RecO, 26 kDa	RecF, – 40 kDa	RecR, 22 kDa
O	1. Monomer in solution 2. Binds both ssDNA and ds DNA 3. Promotes renaturation of complementary ssDNA, requires Mg^{+2}, inhibited by 160 mM NaCl – ATP independent. *(50)* 4. Strand assimilates short oligo and supercoiled DNA, requires Mg^{+2}, ATP independent. *(49).*		
F	1. RecF co-precipitates RecO. This is reversed in presence of ATP *(27).* 2. RecF interacts with RecO even if SSB is present *(27)* .	1. Binds ssDNA, ATP independent *(25, 52).* 2. Inhibits RecA joint molecule formation and RecA ATPase activity *(52).* 3. Binds ATP, dsDNA binding is ATP or ATPγS dependent *(53)* Binding is stronger for ATPγS than ATP. 4. Binding ssDNA or dsDNA or dsDNA enhances ATP binding *(53).* 5. ATPase activity with dsDNA (105). 6. Binding dsDNA with ATPγS – 1 monomer per 4–6 bp *(105).*	
R	1. RecR and RecO overcome SSB inhibition of RecA catalyzed joint molecule formation *(97)* 2. RecOR-SSB ssDNA complex helps RecA to nulceate on DNA *(98)* 3. Stabilzes 5′ end-dependent dissociation of RecA filament *(85)*	1. RecR stablizes RecF on dsDNA in presence of ATP – 1:1 molar ratio of proteins coats dsDNA (105). 2. ATPase activity is stimulated by RecR *(105).* 3. RecR-RecF complex limits extension of RecA filament from ssDNA in a gap to the dsDNA *(106).*	1. *E. coli* protein – No reported activity by itself on dsDNA *(105).* 2. *B. subtilis* protein – Binds ssDNA and ds DNA – binding enhanced by presence of damage in DNA, ATP and divalent metal ions *(3).* Binding of RecR multimers associated with DNA loops in EM *(7).*

partially suppress the UV^S caused by *recA* mutations *(49)*. This correlates with the finding that RecO can catalyze a similar reaction to RecA in vitro (see Subheading 4.2; Table 1; and *49*). Wild-type *recF* overexpression causes inhibition of UV-induced SOS expression, decreased UV^R and cell viability *(79)*. All known mutant *recF* genes show the same phenotypes as a *recF* null mutant when in single copy on the chromosome, but each displays only a subset of the overexpression phenotypes, indicating that the different mutations remove different subsets of RecF activities. Hence, analysis of overexpression phenotypes is a useful method to define and correlate in vivo and in vitro activities of RecF. The *recF4115* mutation is the only *recF* missense protein where all overexpression phenotypes have been eliminated *(74)*.

3.2. Evolutionary Conservation of recFOR Genes

Chromosomal mutations in either the *recF, recO,* and *recR* genes have been most intensively studied in *E. coli* (FOR) (for example, *see [78]*), *S. typhimurium* (F) *(24,60)* and *Bacillus subtilis* (FR) *(2–4)*. Several missense mutations have been isolated in *recF (recF143, recF4101, recF4104, recF4115) (74,79),* insertion mutations (*recF400::kan [94]* and *recF332::Tn3* [9]) and deletions *(recF349) (79)*. Only insertion mutations have been reported so far for *recR (55)* and *recO (33)*.

recF, recO, or *recR* homologs have been reported in 48 different types of bacteria spanning the kingdom of *Proteobacteria* to *Aquicales* (*see* Table 2). Many organisms have homologs of all three genes. In several of the completed genomic sequences only *recF* and *recR* homologs are reported. However, *Helicobacter pylori* and *Aquifex aeolicus* contain only a *recR* homolog and *Borrelia burgdorferi* and *Mycoplasma genitalium* lack *recFOR* homologs. It is worth equal note that while *Treponema pallidum* contains both *recF* and *recR* homologs, this organism has no *recBCD* homologs (and *Borrelia burgdorferi* has the converse:, only *recBCD* homologs and no *recFOR* homologs) *(22,23)*. These findings raise many questions. For example: Do organisms that only have *recF* and *recR* contain only a subset of RecFOR activities? If not, do they have an evolutionary nonhomologous (but functional analogous) *recO* gene? Are organisms without a full RecFOR complement more sensitive to DNA damage than ones with a full complement? It is also noteworthy that only two groups of proteobacteria contain *recO* homologs. From this observation and given the phylogeny of 16S rRNA, it is tempting to speculate that *recO* genes were a late acquisition in the evolution of bacterial-repair systems. Answers to these questions may take some time because little is known about how these diverse organisms repair, replicate, and recombine their DNA.

No *recFOR* evolutionary homologs have been reported in the *Archaea* or *Eucarya.* Rad52, Rad55, and Rad57 in yeast are reported to assist the yeast RecA homolog, Rad51 compete for ssDNA coated with RPA (SSB analog) *(92,93)*. Hence it is possible that while the *recFOR* homologs are not found in eucaryotes, their function is conserved.

3.3. Location and Regulation of E. coli recF, recO, and recR Genes

In bacteria, clues to biological functions of some genes can be suggested by the function of neighboring genes and patterns of transcriptional regulation. In *E. coli,* both *recF* and *recR* are in groups of genes that are needed for DNA replication. The *recO* gene is found downstream of the *era* gene. This gene of unknown function is essential

Table 2
Organisms Containing *recF, recO* and *recR* Homologs

Bacterial grouping	Organism	*recF*	*recR*	*recO*
α *Proteobacteria*	*Caulobacter crescentus*	U37793		
	Rickettsia prowazekii[a]		X[b]	
β *Proteobacteria*	*Neisseria gonorrhoeae*	X	X	X
	Neisseria meningitidis		X	X
	Bordetella pertussis		X	X
	Thiobacillus ferrooxidan	X	X	
γ *Proteobacteria*	*Escherichia coli*[a]	K02179	M38777	U36841
	Haemophilus influenzae Rd[a]	U32780	U32727	U32718
	Haemophilus ducreyi		AF017750	
	Pseudomonas aeruginosa	X	X	X
	Pseudomonas putida	X62504		
	Salmonella typhimurium	X62505	X	U48415
	Proteus mirabilis	M58352		
	Actinobacillus pleuropneumoniae	X63626		
	Actinobacillus actinomycetemcomitans	X	X	X
	Yersinia pestis	X	X	X
	Shewanella putrefaciens	X	X	X
	Pasteurella multocida	X	X	X
	Klebsiella pneumoniae	X	X	X
	Vibrio cholerae	X	X	X
	Azotobacter vinelandii	X86404		
	Coxiella burnetii			L27436
ε *Proteobacteria*	*Helicobacter pylori*[a]	AE000602		
	Campylobacter jejuni	X		
Firmicutes (Low GC gram positive)	*Bacillus subtilis*[a]	X02369	X17014	
	Lactococcus lactis	X89367		
	Staphylococcus aureus	X71437	X	
	Clostridium difficile	X	X	
	Clostridium acetobutylicum	X	X	
	Enterococcus faecalis	X	X	
	Streptococcus mutans		X	
	Streptococcus pneumoniae	X	X	
	Streptococcus thermophilus		P96053	
	Streptococcus pyogenes	U07342		
Actinomycetes (High GC gram positive)	*Mycobacterium smegmatis*	X92503		
	Mycobacterium tuberculosis[a]	Z80233	AL022121	
	Mycobacterium leprae[a]	Z70722	AL023596	
	Mycobacterium bovis	X	X	
	Streptomyces coelicolor	L27063		
Green sulfur	*Chlorobium tepidum*	X	X	

(Continues)

Table 2
Continued

Bacterial grouping	Organism	*recF*	*recR*	*recO*
Cyanobacteria	*Synechocystis sp. PCC6803*[a]	D90907	D90916	
Spirochaetales	*Treponema pallidum*[a]	AE001185	AE001268	
Cytophagales	*Porphyromonas gingivalis*	X	X	
Chlamydiales	*Chlamydia trachomatis*[a]	AE001282	AE001297	
	Chlamydia pneumoniae[a]	X	X	
Thermus/Deinococcus	*Deinococcus radiodurans*	X	X	
Aquificales	*Aquifex aeolicus*[a]		AE000742	

[a] Completed genome sequence.
"X" indicates either a partial or full sequence is available in the uncompleted genome database as of August 1999. Genbank accession numbers are given where known.

for growth. As will be expanded upon later, the association of *recF* and *recR* with DNA replication genes is suggestive that these two proteins (and possibly *recO* as well) may interact with the replication machinery.

Understanding of the transcriptional regulation of the three groups of genes encoding *recFOR* is at a rudimentary level. Most is known about the transcriptional regulation of the *recF* gene. Transcriptional studies using different fragments of the *dnaA-dnaN-recF* region fused to *lacZ* on both plasmids and chromosomes have identified several sequences that act as promoters and transcriptional terminator *(5,6,68).* These results suggest that this region of the chromosome is under complex regulation and that *recF* transcriptional regulation is part of a larger network involving *dnaA* and *dnaN (11,29).* It has been recently shown that *dnaN* and *recF* promoters are induced greater than 40-fold during entry into stationary phase *(100).* Studies on the transcriptional regulation of the *recO* and *recR* genes have not yet been reported.

The level of the *recFOR* proteins in the cell is thought to be quite low, although it has not been precisely determined. This speculation is based on several observations. First an upper limit of the amount of RecF has been estimated at less than 190 molecules per cell *(52).* Second, several processes that inhibit *recF* overexpression were identified when *recF* was overexpressed from a plasmid *(76,77).* Third, the codon usage of *recF* is similar to that of other poorly expressed *E. coli* genes *(9).* Although overexpression of *recR* is not problematic like *recF,* expression of *recR* in maxicells is quite low *(56).* Another interesting observation is that the *recF, recO,* and *recR* genes all overlap with the genes in upstream of them. This type of arrangement, called translational coupling *(67),* is used by the cell to ensure equal levels of expression of two proteins that interact. In the case of *recF* and *recR,* however, a mechanism opposite of translational coupling seems to operate because much less of the *recF* and *recR* gene products are seen relative to the *dnaN* and *orf12* gene products, respectively *(9,55).* A common mechanism acting either at the level of transcription or translation to regulate or coordinate levels of *recFOR* gene expression remains to be elucidated.

4. BIOCHEMICAL INTERACTIONS BETWEEN RecFOR AND OTHER PROTEINS

4.1. Interactions with SSB and RecA

The idea that *recFOR* modulates the binding of SSB and RecA to ssDNA derives from both genetic and biochemical data. Moureau *(61)* showed that overexpression of SSB yielded phenotypes similar to those of *recF* mutants. Volkert and colleagues *(102,104)* isolated several suppressors of *recF* mutations that mapped in *recA* (e.g., *recA803*). Others showed that these suppressors would also suppress *recO* and *recR* mutations *(52,103).* Biochemical analysis showed that RecA803 protein could make joint molecules under conditions where SSB was inhibitory to RecA-catalyzed reactions *(54).* Umezu et al. *(97)* showed that RecO and RecR were sufficient to overcome the SSB inhibition in RecA-catalyzed joint molecule reactions. The unresolved finding in this report was that RecF had no role in the in vitro reaction. This was in contradiction to the in vivo reaction, where *recF* has a definite role. At least one aspect of the reaction conditions in these experiments did not mimic the in vivo conditions as very high levels of RecO and RecR were used in vitro and only low amounts of RecOR are thought to present in vivo. This prompted further testing, which showed that overexpression of *recOR* could suppress *recF* mutations and provided a reason for why RecF was not needed in the in vitro reactions *(78).* These findings led to the molecular matchmaker model for RecFOR function in *E. coli (14,78).* This model proposes that RecF acts as a molecular matchmaker identifying and binding to a specific DNA structure: a gapped DNA intermediate left by DNA replication where SSB was bound to ssDNA. RecF would then help to load RecOR, which in turn helped to modify the SSB-ssDNA so that RecA could bind *(14,78).*

4.2. RecFOR Biochemistry

Biochemical analysis of the RecFOR proteins has been extremely difficult for at least three reasons: (1) There are no known enzymatic activities to follow during purification. Hence the proteins are purified on the criteria of solubility and electrophoretic purity. (2) The structure of the DNA substrate on which RecFOR operates is not known. It is, however, hypothesized to be a gapped DNA molecule produced by DNA replication. (3) Because DNA replication is required for RecF-dependent SOS induction *(84),* it is likely that in vitro visualization of RecFOR activity will require components of the DNA replication machinery. In spite of these difficulties, significant achievements have been made in understanding the biochemical properties of these proteins. All three proteins have been overproduced and purified. Table 2 lists the known activities of the proteins both singly and in combination.

Singly, the *E. coli* RecO and RecF and *B. subtilis* RecR proteins display properties of proteins that are likely to be involved with DNA metabolism. They bind both ssDNA and dsDNA and this binding is modulated by divalent metal cations and nucleotide cofactors. RecO has two activities that could be specifically associated with recombination: renaturation of ssDNA and strand assimilation of an oligonucleotide with a homologous dsDNA circular supercoiled DNA substrate. Like RecO, the yeast *RAD52* protein also has an ATP-independent strand-transfer activity *(66).*

Early in its study, RecF activity was defined in terms of its ability to inhibit RecA catalyzed reactions *(52).* Although these are interesting reactions in their own right, one

needs to remember that in vivo there is usually a large excess of RecA over RecF. Therefore, these reactions may not be physiologically relevant. More recently, conditions for an ATPase activity have been identified for RecF in the presence of dsDNA *(105).* It is likely that this activity is physiologically relevant and important. Evidence supporting this idea includes: (1) *RecF4101,* a mutant in the phosphate binding hole, is defective in RecF activity in vivo *(75).* (2) RecF4101 does not display ATPase activity in vitro *(105).* The RecF ATPase activity is also stimulated in the presence of RecR *(105).*

RecOR and RecFR display activities that modulate the activity of RecA protein. RecOR help RecA load onto ssDNA coated with SSB, as previously mentioned. They also help to stabilize RecA filaments and prevent end-dependent dissociation *(85).* These two mechanisms may be related. It is easy to visualize how these two activities would be useful in the early stages of RecA filament formation. The effect of RecFR on RecA protein filaments was discovered by first looking at the effect of RecR on RecF binding to dsDNA. RecF binds to dsDNA in a sequence-independent fashion. Binding is stronger in the presence of ATPγS than ATP. RecR stabilizes RecF binding to dsDNA in the presence of ATP *(105).* When RecA binds to ssDNA on a gapped DNA substrate, eventually RecA filament formation will extend beyond the ssDNA into the dsDNA region. If RecFR is present, it will halt growth of the RecA filament *(106).*

Madiraju and colleagues have defined complexes of RecFOR and SSB proteins in the absence of DNA using immunoprecipitation and different types of chromatography *(27).* They find that RecO interacts with RecF, RecR, and SSB. In these assays, RecO can bind either RecR or SSB but not both. On the other hand, Umezu and Kolodner found using BIAcore sensor chips that RecO can bind both RecR and SSB and that SSB binds RecO with higher affinity than RecR *(98).* The former group found that RecO can bind RecF and SSB at the same time and that the addition of ATP abolishes the RecFO interaction. They also see complexes between RecFOR and RecFOR-SSB depending on the order of addition.

In summary, it appears that the RecFOR proteins are capable of a variety of activities in vitro either singly or in combination that could be useful in recombination. Whether any of these activities is used by these proteins in vivo remains to be proven.

5. A MODEL FOR THE ROLE OF RecFOR IN THE CELL

It is arguable that the main function of recombination is to help restart stalled replication forks *(17,18,36,99).* The process can be envisioned in the following steps: detection of DNA damage by the replication machinery, production of a gap in the DNA behind the replication fork, PRR substitution of DNA not replicated because of damage (e.g., RecA, RecFOR), and then restarting of the replication machinery. Several observations mentioned previously are consistent with RecFOR having an important role in the repair of collapsed and/or stalled replication forks by recombinational DNA repair. It is conceivable that RecF (OR) persists during the entire repair process and is not necessary only during the early pre-synaptic phase.

Figure 4 shows a model that combines several of the ideas represented in the literature *(14,17,27,78).* The model focuses on the role of the RecFOR, RecA, and SSB proteins in PRR. The model assumes that the replication fork encounters a noncoding lesion on the template for the leading strand. The replication fork leaves a gap and pauses or stalls at least one Okazaki fragment upstream. The ssDNA is first covered

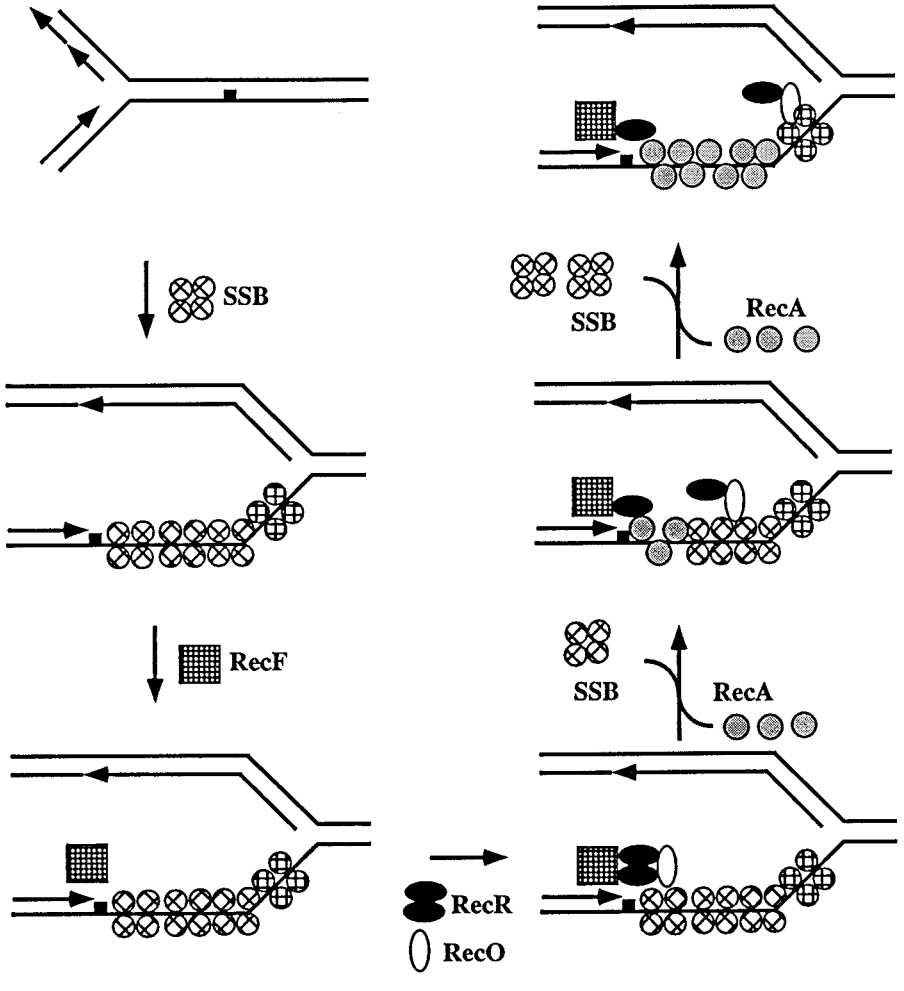


Fig. 4. Model of RecFOR in PRR. In the first panel, a replication fork approaching a noncoding lesion (small black box on the DNA) is diagrammed. In the second panel, the replication fork has translocated one Okazaki fragment past the noncoding lesion. No replication has occurred on the template strand containing the noncoding lesion. The remainder of the diagram is explained in the text.

with SSB. RecF then recognizes the DNA-protein structure (assumed in this diagram to be at the left edge of the gap) and then helps to load RecO and RecR. RecR is shown as a dimer at this point. After RecFOR assembly, the complex splits. One subunit of RecR goes with each RecO and the other RecR subunit remains with RecF. The RecOR complex is free to interact with SSB and help load RecA. When it reaches a dsDNA section, it stops and anchors the 5′ end of the RecA filament preventing dissociation. The RecFR complex left behind prevents the RecA filament from extending in to the dsDNA and focuses the RecA filament in the region of ssDNA. The RecA filament then searches the other daughter duplex for a region of homology and places the noncoding lesion across from its complement. The DNA lesion is now ready for removal by an excision repair reaction.

This model incorporates many of the observations mentioned in this article. However, it does not indicate how RecOR moves along the DNA removing the SSB, helping RecA to bind to the ssDNA or define when the RecF ATPase activity is needed. It also has RecA loading in a discontinuous fashion (3′ to 5′) *(69).* An alternative scheme (not shown) that does not require RecOR to migrate along the DNA suggests RecR protein stabilizes a loop of ssDNA (as has been shown for the *B. subtilis* RecR protein) such that ends of the ssDNA region are close together. The complex can then split into RecFR and RecOR subcomplexes attached to each end of the gap and function as explained earlier. This scheme also has the advantage that RecA can load continuously in the 5′ to 3′ direction from the RecOR nucleating point.

5.1. A Molecular Connection Between RecF(OR) and DNA Replication

The position of *recF* and *recR* on the chromosome in the middle of operons encoding DNA-replication proteins and the observation that UV-induction of the SOS response by *recF* requires DNA replication suggests a connection between *recF, recR,* and DNA replication. Rothman and Clark *(70)* showed that after UV irradiation, DNA synthesis in a *recF143* mutant was greatly reduced relative to wild-type. Courcelle et al. *(16)* have shown that both *recF* and *recR* mutations lead to much greater amounts of degradation of newly synthesized DNA after UV irradiation and a decreased ability to complete ongoing rounds of DNA replication than wild-type.

A role for *recF* in DNA replication has been suggested by overlapping activity with *priA* in UV-induction of the SOS response (pre-synaptic role) and cell viability (post-synaptic role) *(73).* PriA, originally isolated as a component of the ΦX174 in vitro DNA replication system (a model for primosome assembly and synthesis of RNA primers during lagging strand DNA synthesis at a replication fork [58]) has been shown to be essential for DNA repair and homologous recombination *(32,82).* Interestingly, the overlapping roles of *priA* and *recF* are not shared by *recR* and *recO.* This is the only example of a phenotype where *recF* is different from that of *recO* and *recR* (where all three have been tested). It should be noted that the proposed post-synaptic role for *recF* is highly speculative.

Three additional observations support a role for *recF* in DNA replication and or cell viability. The first is that *recF143* causes a decrease in UV-mutagenesis with ssDNA phages *(12).* This function may be overlapping with *priA.* The second is that *recF* is necessary for stable DNA replication and cell viability in a *rnh-102 recA200 rin-15* and *rnh-102 recA200 rin-15 dnaA508* strains, respectively *(95).* The third situation in which *recF* may have a role in DNA replication is that it is needed for viability in a *recA200 polA25::spc lexA71::Tn5* strain *(10).* Although a common thread between the latter three mutant strains is not apparent, all have a defect in DNA replication (and or cell viability) that is suppressed by some mutation (either *rin-15* or *lexA71::Tn5*) and this new situation is then dependent on the *recF* gene product. It is not clear, however, if *recF* participates in a pathway that is active to a small degree in wild-type cells and this becomes the major pathway in the mutant cells, or if the pathway only becomes active in these "suppressed" states. The dependence of *recO* and *recR* in these strains has not been addressed.

6. THE ROLE OF THE PRIMOSOME ASSEMBLY PROTEINS IN RESTARTING REPLICATION FORKS

The role of the primosome assembly proteins (PriA, PriB, PriC, DnaB, DnaC, DnaG, DnaT) in the cell is beginning to become clear (reviewed in *[57,58,80,81]*). These *E.*

coli proteins were originally discovered as host proteins required in the ΦX174 in vitro DNA replication system. The biochemical properties of these proteins suggested that they were involved in the synthesis of RNA primers on the lagging strand at a replication fork. Although this is still possible, it now appears that these proteins have different or additional roles. It is now believed that these proteins help to restart replication forks that have stalled or collapsed and were repaired by recombinational and RecA-independent processes.

6.1. Primosome Assembly Proteins in DNA Replication In Vitro

Although the ΦX174 in vitro DNA replication system may not be the best model of the in vivo function of the primosome assembly proteins, this system provides the clearest picture at the biochemical level and provides a platform for discussing their in vivo functions. A key feature of the assembly process is the DNA substrate to which the proteins are loaded. This is the primosome assembly site (PAS) of the ΦX174 chromosome. Although other ssDNA phages and some plasmids have these sites, they have never been found on the *E. coli* chromosome. It is thought that both sequence and secondary structure are important for PAS *(1,89).* It is suspected that the PAS is a structure that some phages and plasmids have evolved to take advantage of the *E. coli* host system for their replication. Figure 5 shows the order of assembly of the primosomal proteins onto the PAS *(62,63).* The PriA protein binds to PAS and serves as a platform for the loading of the other proteins. PriA is a multifunctional protein with helicase, ATPase, and translocase activities that are genetically separate from its ability to assemble primosomes *(109).* The PriB protein binds to PriA-PAS. It is thought that PriB stabilizes PriA at PAS *(41).* DnaT then loads onto the PriA-PriB-PAS complex. These three proteins form a protein-nucleic acid complex that serves as an entry point for DnaC to load DnaB, the replicative helicase. DnaC is the only primosome assembly protein not part of the final primosome. Using some DNA substrates with PAS, there is an optimizing requirement for PriC before this step. However, the role of PriC is unknown. The PriABC-DnaTB complex is called the pre-primosome and DnaG (Primase) can interact with it in a distributive *(108)* fashion to synthesize RNA primers that are competent to be extended by Pol III holoenzyme.

6.2. Primosome Assembly Proteins and E. coli Replication Restart

Many questions were raised that eventually helped to clarify the role of the primosome assembly proteins in *E. coli.* The first is based on the observation that no PAS sites have been found on the *E. coli* chromosome. If true, then what is the natural substrate for PriA? Also, no slow or fast stop mutants of *priA, priB,* or *priC* have ever been isolated and none of these proteins are needed in an in vitro system that mimic initiation of DNA replication at *oriC.* This raised questions about how and when PriA PriB, DnaT, and PriC become associated with a replication fork. Surprisingly, a *priA* null mutant was unexpectedly found to be viable, deficient in recombination and DNA repair, and had high basal levels of SOS expression *(39,65).* Hence PriA was a DNA replication protein that also had roles in recombination. Tokio Kogoma *(30–32)* proposed that the pathway of primosome assembly might be used by *E. coli* to load replication forks at recombinational intermediates. This hypothesis has led to many advances in understanding the biochemistry of PriA. Several studies *(40,59,64)* have now shown that PriA binds to D-loops, a key recombinational intermediate.

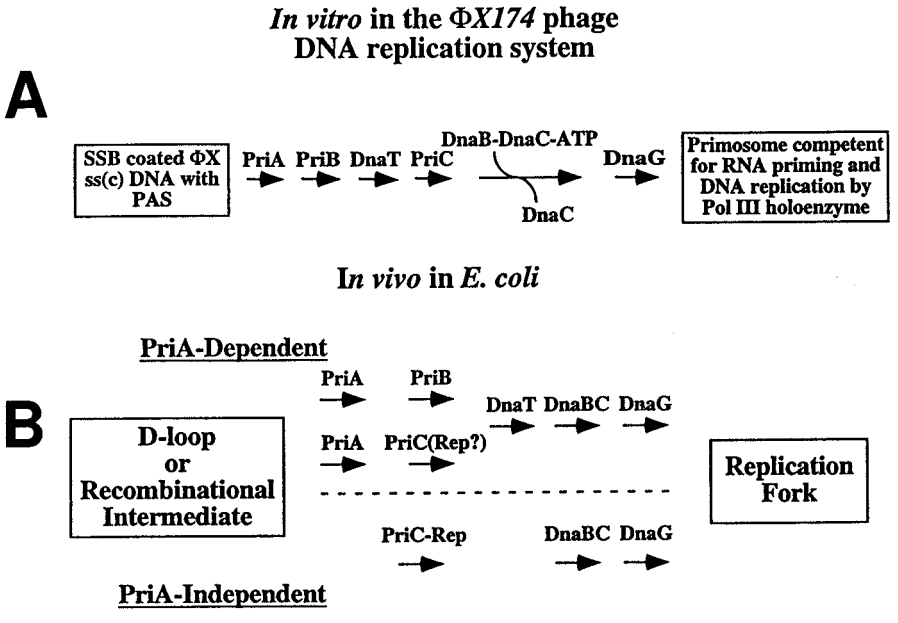


Fig. 5. Two models for the action of the primosome assembly proteins. In **A,** the in vitro assembly of the primosome assembly proteins on the ssDNA phage is shown. In the **B,** is a model for two pathways of primosome assembly as a prelude to the loading of a DNA replication fork. One should note that in the bottom half, although the substrate is referred to as a recombinational intermediate like a D-loop, in actuality this substrate has yet to be identified in vivo. The Rep protein in the PriA-dependent pathway is listed with a question mark because its role is currently not clear.

Understanding the genetics of the primosome assembly genes have been of intense interest to our lab. Almost every simple prediction of ΦX174 model so far has not proved true for *E. coli.* For instance, one would predict that *priB* and to lesser extent, *priC,* should have the same mutant phenotypes as *priA* mutants, but this was not the case. Null mutants of *priB* and *priC* are not readily distinguishable from wild-type *(81).* Yet the *priB priC* double mutant is inviable, suggesting that these proteins have a redundant and essential role in *E. coli.* Extragenic suppressors of *priA* mutations have been found and mapped in *dnaC (82).* These are thought to load DnaB at the correct DNA substrate in the absence of PriA, PriB, PriC and DnaT. While these *dnaC* suppressors fully suppress *priA* mutant phenotypes, they only partially suppress *priB priC* mutant phenotypes *(81).* In vitro, the PriA suppressor DnaC810 can load Pol III holoenzyme (via DnaB) at a D-loop *(42).* Hence the biochemistry is beginning to agree with the in vivo data.

Finally, a lingering question has been why *priA* mutations are not lethal, as it would seem that restarting replication forks at recombinational intermediates is essential. One idea is that there are multiple pathways for restarting replication forks. Evidence for this has come from synthetic lethality studies of pairs of primosome assembly mutants *(71).* Figure 5 shows a diagram of one model that explains how some gene products may be involved in these multiple pathways. It is noteworthy that the *priC* protein, which has not found a secure home in the ΦX174 model, is essential for the PriA-independent path-

way. Also, another orphan DNA replication gene, *rep,* is essential for this pathway. Additional experiments show that the *dnaC809* suppression pathway of *priA* mutant phenotypes occurs by the elevation or modulation of the PriC-Rep pathway *(71,80).*

Why would *E. coli* two pathways to restart replication? Although there are several possible reasons, the most appealing is that there are different DNA substrates that need to be processed into replication forks. These could arise by different mechanisms such as replication fork arrest or collapse. One might expect that repair of these two situations would lead to different DNA structures with different complements of proteins and thus may be optimally restarted by two different systems. This idea is analogous to the RecBCD and RecF pathways of recombination, which act preferentially on different types of substrates (double-strand ends or gaps) to repair and recombine DNA.

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3

Abasic Site Repair in Higher Eukaryotes

Phyllis R. Strauss and Noreen E. O'Regan

1. INTRODUCTION

Base-excision repair (BER) refers to a repair pathway that generates and repairs abasic sites in double-stranded (ds) DNA (Fig. 1) *(101,134,222,264).* BER is important not only in maintaining the integrity of nuclear DNA but also in protecting mitochondrial DNA against oxidative onslaught from $FADH_2$ and NADH and the reactive oxygen species generated during O_2 reduction *(42).* Estimates of the number of abasic sites generated per mammalian cell per day run as high as 10^6/cell/d *(88).* Abasic sites are unstable, degrading spontaneously into DNA strand-breaks by β-elimination *(132)* that retard DNA polymerases *(43,44,50,66,91,237).* They are highly mutagenic because of nontemplated DNA *(59,108,273)* and RNA *(66,216,217,283)* synthesis. Moreover, abasic sites engage in suicide reactions with topoisomerase I, leading to permanent DNA damage and premature cell death *(196)* and can form covalent complexes with topoisomerase II that cause DNA double-strand breaks *(107),* which can bind poly (ADP-ribose) polymerase *(2,152,153).* Despite the large number of abasic sites generated per cell per day, the number of resulting mutations is extremely low. The difference reflects the elaborate mechanisms that the cell has devised to repair abasic sites *(134).*

An abasic site can be created by spontaneous base loss as in depurination, by DNA oxidation *(133,134,250),* or by the action of DNA glycosylases *(45a,66,115,159),* which recognize and cleave nonbulky base lesions in DNA *(115).* Some glycosylases remove the altered base without cleaving the DNA backbone. Others not only remove the base and but also cleave the abasic site 3′ to the sugar residue (Fig. 1). Cleavage 3′ to the sugar residue occurs by a lyase or β-elimination mechanism *(115,159).* In either case, once the abasic site has formed, it must be nicked on the 5′ side of the dRP in order to generate the free 3′ hydroxyl group that is required by the repair polymerase to insert the correct nucleotide(s). Depending on which polymerase inserts the correct nucleotide(s), the strand downstream of the abasic site may remain undisturbed or it may be subject to strand displacement. Subsequently, the dRP residue and any displaced DNA are removed so that the newly reconstructed strand can be ligated.

The enzyme that appears to be common to all the branches of the BER pathway is AP endonuclease (AP endo). AP endo cleavage generates the critical free 3′ hydroxyl for repair DNA synthesis *(58).* Because all the repair mechanisms converge at the aba-

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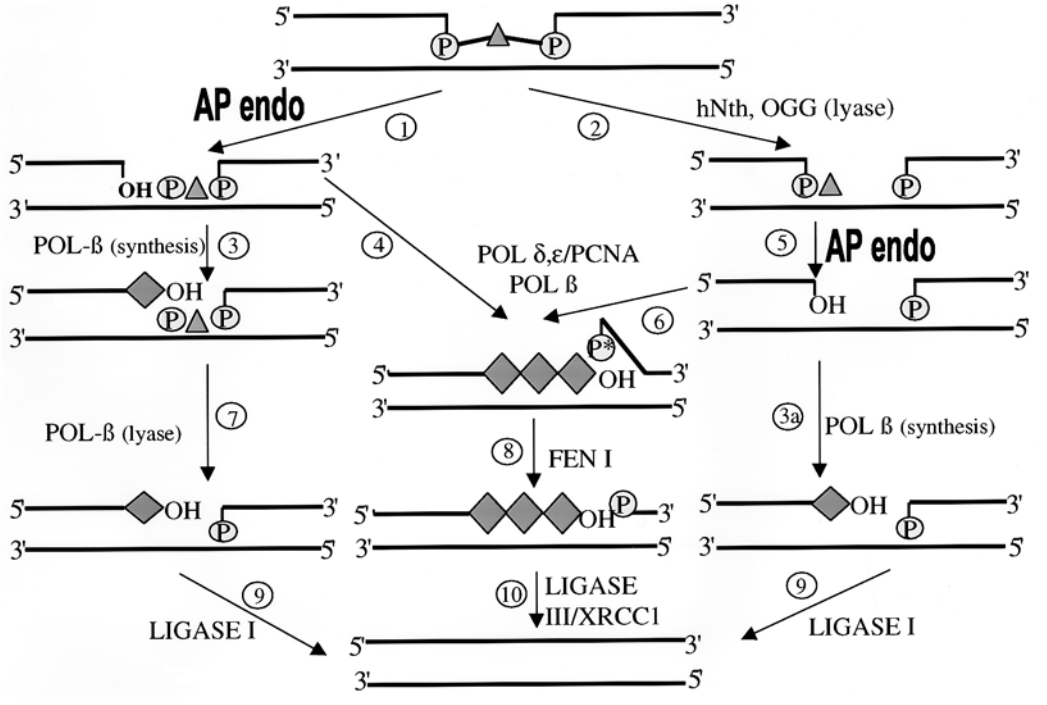


Fig. 1. Repair of abasic sites diverge after the generation of the 3′-hydroxyl required for replacement synthesis. The various enzymatic reactions are numbered for reference to the text. P, phosphate; Δ, deoxyribose; ◇, newly inserted nucleotide; P*, 5′-deoxyribose phosphate. The product of displacement synthesis (reactions 4 and 6) can be 2–6 nucleotides in length but is shown as 3 nucleotides for clarity. Reactions 9 and 10 may be interchangeable. Short-patch repair is comprised of steps 1 or 2, 3 or 5, 7 or 3a, and 9 or 10. Long-patch repair is comprised of steps 1 or 2 and 5, 4 or 6, 8, and 9 or 10.

sic site and because the repair processes diverge after cleavage of the abasic site 5′ to the phosphodeoxyribose, we suggest that this repair pathway is better termed abasic site repair (ASR). Henceforth, we will refer to the pathway as ASR.

This review will present a detailed biochemical picture of the overall ASR pathways in higher eukaryotes and then review the enzymes and the auxiliary proteins involved in the individual steps. After presenting the evidence for interactions among the components involved in ASR, we discuss subcellular locations of the pathway and, finally, probable interactions with other DNA repair systems. We shall briefly discuss the different DNA glycosylases without going into detail, because they are involved in generating the abasic site but not repairing it and because they have been reviewed extensively elsewhere *(45,45a,115,159).* Studies on yeast are omitted because the various enzymatic activities found in higher eukaryotes, while present, may be grouped differently and are reviewed elsewhere *(4,98,219,279).* The major emphasis will be on studies appearing in the last several years.

2. OVERVIEW

Under normal circumstances, DNA undergoes depurination at a measurable rate such that some 10,000 abasic sites/mammalian cell/d are generated *(66,133,134).* In

addition, a variety of lesions in DNA arise by spontaneous deamination of cytosine, from errors occurring during replication including insertion of damaged bases or of uracil, from reactions with endogenous reactive oxygen species *(50,66,162)* or from exogenous exposure to toxic species in food and/or the environment *(66,162).* The lesions include, among others, uracil, 3-methyladenine, 8-oxoguanine, and thymine glycol. Nonbulky lesions are generally recognized by the DNA glycosylases that remove the modified base and leave an abasic site (Fig. 1). Higher eukaryotic cells have two classes of DNA glycosylases, those that remove the base only and those that remove the base and nick the DNA on the 3′ side of the dRP residue. Enzymes in the first category include uracil DNA glycosylase (UDG), G/T glycosylase, thymine glycol-DNA glycosylase, thymine DNA glycosylase, and 3-methyladenine-DNA glycosylase *(115).* UDG is by far the most abundant and active of these, because the presence of uracil in DNA occurs at high rates. Under normal circumstances estimates range as high as one deoxyuridine out of every 200 or 300 thymidine residues inserted during replication *(112).* Another major source of uracil in DNA is the spontaneous deamination of cytosine *(66).* The second set of glycosylases include 8-oxoguanine DNA glycosylase (OGG), hNth1 *(92),* N-methylpurine/DNA glycosylase *(66),* and adenine-specific DNA glycosylases *(112).* N-glycosylases in eukaryotic cells are not abundant *(115,222).* To complicate matters further, at least one DNA glycosylase is able to remove normal bases in a random fashion, albeit at a slow rate *(15).*

In most instances, apurinic/apyrimidinic endonuclease (AP endo) initiates the repair process of an abasic site (Fig. 1, reaction 1). AP endo cleaves an intact abasic site 5′ to the phosphodeoxyribose (dRP) and generates the 3′ hydroxyl to be used by the DNA polymerase that will insert the correct nucleotide. The dRP moiety remaining attached to the downstream strand will be removed in subsequent steps. If a glycosylase/lyase has already nicked the abasic site 3′ to the dRP (reaction 2), AP endo has been proposed as the enzyme that facilitates removal of the 3′ phosphodeoxyribose residue (reaction 5) and generates the all-important 3′ hydroxyl for subsequent polymerase replacement of the nucleotide (reaction 3a) (but see section on AP endo later).

If the glycosylase has not nicked the abasic site 3′ to the dRP residue, DNA polymerase β (pol β) fills the gap with the correct nucleotide (reaction 3) and then excises the dRP from the downstream cleaved strand (reaction 7). Because synthesis is limited to a single nucleotide, this pathway is commonly called "short-patch" or "single-nucleotide" BER. We shall refer to it as short-patch ASR. Ligase I or possibly ligase III together with XRCC1 (*X*-ray Cross-Complementation protein 1) then seals the two ends together (reaction 9). In the alternate pathway (reactions 4, 6, 8, 10), pol β *(55)* or DNA polymerase ε or δ in conjunction with Proliferating Cell Nuclear Antigen (PCNA) replaces the missing nucleotide plus 2–6 nucleotides downstream of the initial abasic site (reaction 4 or 6). The displaced strand is cleaved by flap endonuclease (FEN1) (reaction 8). Because 2–6 nucleotides are inserted after the 3′ hydroxyl, this pathway has been referred to as "long-patch" or "alternate" BER *(47,57,109,200)* in contrast to short-patch repair where only the one nucleotide gap is filled. After long-patch repair, ligase III in conjunction with XRCC1 or possibly ligase I seals the ends of the DNA strand together (reaction 10). Some reports indicate that the ligases (ligase I and ligase III/XRCC1) may be interchangeable *(109).*

The biochemical mechanism for deciding which pathway is used for insertion of nucleotides and rejoining is speculative at this time. Long-patch repair in some cells is

observed when pol β is deficient *(16,63)* or when the dRP residue cannot be cleaved by lyase activity, as occurs when the abasic site is reduced *(109)* (*see* below). In other pol β-deficient cells, long-patch repair is not detected *(215)* unless a closed circular substrate is employed *(55).* To complicate matters further, antibody against pol β inhibits long-patch repair in cell-free extracts *(109).* In a reconstituted system described by Fortini et al. *(64) (see Subheading 2.4.),* the choice of pathway depended on the type of lesion. Repair of lesions requiring a DNA glycosylase/lyase was restricted to short-patch ASR, whereas repair of lesions requiring a DNA glycosylase without lyase activity could be repaired by either long- or short-patch ASR. Whether these are the only situations where each pathway occurs is not known.

The various reaction sequences of ASR have been measured in extracts from a variety of higher eukaryotic cells including *Xenopus laevis* oocytes *(154,156),* bovine testis *(225),* mouse fibroblasts *(36,227),* and human cell lines *(52,54,56,109,175).* Apparently, short-patch ASR predominates in human and mouse cells when the substrate is a ds oligonucleotide or plasmid substrate containing a G/U mispair *(16,67,109,136,227),* because the inactivation of pol β by blocking antibody prevents the completion of repair *(109,227)* and because knockout mouse cells lacking pol β are unable to perform ASR on a short oligonucleotide *(227)* (but *see* above Subheading 2.2.2.). In addition, extracts from fibroblasts that respond to lipopolysaccharide treatment with increased BER fail to respond if the cells lack pol β *(36).* These results would imply that in extracts from these cells at least the long-patch pathway is lacking, inefficient, or not increased. Reconstitution of long-patch repair by human cell extracts has also been accomplished. The substrate was ds closed circular DNA containing a single uracil *(54,55).* In this case, RPA stimulated PCNA-dependent repair. The two cofactors were additive; neither could substitute for the other.

The reaction sequences for the short- and long-patch pathways have now been reconstituted with purified proteins *(64,109,116,177,229).* Components in the former pathway most often include UDG, AP endo, pol β, and DNA ligase I or DNA ligase III/XRCC1. The substrate is often a defined oligonucleotide containing a G/U pair, with substrate and enzymes being present in approximately equimolar concentrations. The rate-determining step for the short-patch pathway on a 51-mer ds oligonucleotide with a single G/U pair at position 22, i.e., one involving generation of the abasic site by UDG, single nucleotide gap filling by pol β, and ligation by DNA ligase I, has been identified *(229).* The insertion of the new nucleotide can occur prior to removal of the dRP moiety. The enzymatic efficiency (k_{cat}/K_m) of the individual components ranges from 420 $\mu M^{-1}s^{-1}$ for UDG to 0.15 $\mu M^{-1}s^{-1}$ for the dRP lyase activity of β-pol. Indeed, the number of molecules per cell for pol β is about 10% that of AP endo (3.5×10^5 for human fibroblasts and 7×10^6 for HeLa cells) *(34).* In other words, not only is pol β an inefficient enzyme, it is present at ~10% the level of AP endo. Because the slowest step in the pathway is the dRPase activity and because this rate reflects the overall rate of the pathway, the dRPase activity of pol-β is likely to be the rate-limiting step *(229).*

Components in the long-patch pathway reconstituted from purified or recombinant proteins may include UDG, FEN1, PCNA, pol δ and/or pol ε, ligase I or III, RPA or RFC, and XRCC1 *(47,71,109,116,158,165).* Omission of PCNA causes accumulation of pre-excision reaction intermediates following strand displacement, which is overcome by the addition of PCNA *(71).* A PCNA mutant unable to bind FEN1 is unable to stimulate excision *(71).* On the other hand, if pol β performs long-patch repair, as might

occur when its dRPase cannot remove the dRP residue (for example, if the abasic site were created by tetrahydrofuran or had been stabilized by reduction), then FEN1 can still cleave the displaced DNA strand without the assistance of PCNA *(71).* In most cases, RFC is required and RPA will not substitute. However, the choice of factor may depend on the starting substrate. As of this writing, there are no data available for the kinetic measurements of individual components in the long patch pathway.

An interesting series of experiments using mammalian cell extracts examined whether pol β is the *only* polymerase that can perform short patch ASR *(53).* The substrate was a closed circular DNA containing a single 8-oxoguanine at a defined site. Extracts from pol β deficient mouse cells resulted in extension of the repair gap by long-patch repair only 50% of the time. The remainder was accomplished through replacement of a single nucleotide. Thus, in cases where the pathway is initiated via a glycosylase/lyase reaction BOTH long-patch and short-patch repair can occur, *even in the absence of pol* β. Clearly this is a different case than the situation where ASR is initiated through removal of a uracil owing to the action of UDG and suggests that polymerase(s) other than pol β are capable of filling the single nucleotide gap through short-patch repair. Because pol δ is not known to be able to insert single nucleotides, is it possible that under these circumstances pol ε, which is involved in maturation of Okazaki fragments (*see* below Subheading 2.2.2.2.), inserts a single nucleotide, thus performing short-patch repair? Or, in cell extracts such as these, could the polymerase be DNA polymerase α or one of the newly discovered or as yet undiscovered DNA polymerases not previously associated with ASR?

2.1. The Abasic Site

Several NMR studies have examined the structure of the abasic site in solution when the abasic site is located in a ds oligonucleotide *(11,43,44,130A,131,147,253).* In general, the presence of an abasic site increases flexibility of the DNA and concomitantly increases the diffusion constant *(147).* However, the solution structure of the oligonucleotide depends on the base opposite the abasic site and the sequence context. An apurinic site differs from an apyrimidinic site. The former has α and β hemiacetal forms in approximately equal amounts, whereas the latter is predominantly the β hemiacetal. Sequence context is also important, because the NMR structure of an abasic site located in the context of curved DNA (dA tract) is different from the ones just described *(253).* In addition, the structures of the both α and β hemiacetals differ from those where the DNA oligonucleotide is not bent.

In a study investigating the effects of an abasic site on DNA conformation, when a tetrahydrofuran was incorporated into a family of duplex 13-mers, the global B-form conformation did not change. However, the presence of the lesion induced enthalpic destabilization of the duplex with the magnitude of the effect being dependent on the sequence context *(74).* In a second study using a ds 10-mer with a tetrahydrofuran inserted into the center of one of the strands, the stem region of the duplex adopted a right-handed helical structure with the abasic site excluded from the helix *(131).* The effect of the presence of a second abasic site on the contralateral strand is described in Subheading 2.2

Despite local thermodynamic and structural changes in DNA containing an abasic site or an abasic site analog, AP endo, the enzyme responsible for recognizing abasic sites, appears to have little sequence preference *(28,263;* Mckenzie and Strauss, unpublished data). Consequently, it is likely that the enzyme itself is capable of imposing a

Table 1
Proteins Involved in ASR[a]

Repair protein	Subunit	Molecular mass (kDa)	Chromosomal location (Human)
AP endonuclease		35.5	14q11.2–12 *(208)*
The polymerases			
Pol β		39.0	8p11–12 *(27)*
Pol δ	Catalytic	125	19q13.3 *(103)*
	Small	50	7 *(280)*
Pol ε	Catalytic	261	12q24.3 *(235)*
	Small	55	14q13–q21 *(129)*
Pol γ		136	15q24–q26 *(210,252,286)*
Associated enzymes			
FEN1		42	11q12 *(86)*
Ligase I		125	19q13.2–13.3 *(287)*
Ligase IIIa		922	17q11–12 *(35,259)*
Ligase IIIb			" "
Mitochondrial ligase			" "*(121)*
Auxilliary proteins			
PCNA		29	20p12–p13 *(205,245)*
$p21^{Cip1/Waf1}$		21	
RPA	hRPA70	70	17p13.3 *(288)*
	hRPA32	32	1P35 *(288)*
	hRPA14	14	7P22 *(288)*
RFC	p140	140	4P13–p14 *(142)*
	p40	40	7q11.23 *(184)*
	p38	38	13q12.3–q13 *(184)*
p37	37	3Q27 (184)	
	p36	36	12q24.2–q24.3 *(184)*
PARP		122	1q42 *(84)*
XRCC1		70	19q13.2 *(122)*

[a] The individual proteins with their molecular masses and human chromosomal location are listed. The numbers in parentheses refer to the literature citation.

transition state conformation that is independent of the initial conformation of the abasic site-containing oligonucleotide. (*See* also the description of domain mapping and co-crystal studies described in Subheading 2.2.1.)

2.2. The Enzymes

The enzymes and auxiliary proteins that participate in ASR are listed in Table 1 that also includes their subunits, molecular masses, and chromosomal locations in the human genome. A more complete description of each protein follows.

2.2.1. AP Endonuclease

The enzyme that initiates ASR is AP endo *(93).* The enzyme is highly conserved from man to *E. coli,* where the homologous enzyme is exonuclease III (Exo III) *(7,51,58,209,223).* The active site is also structurally similar in these two enzymes.

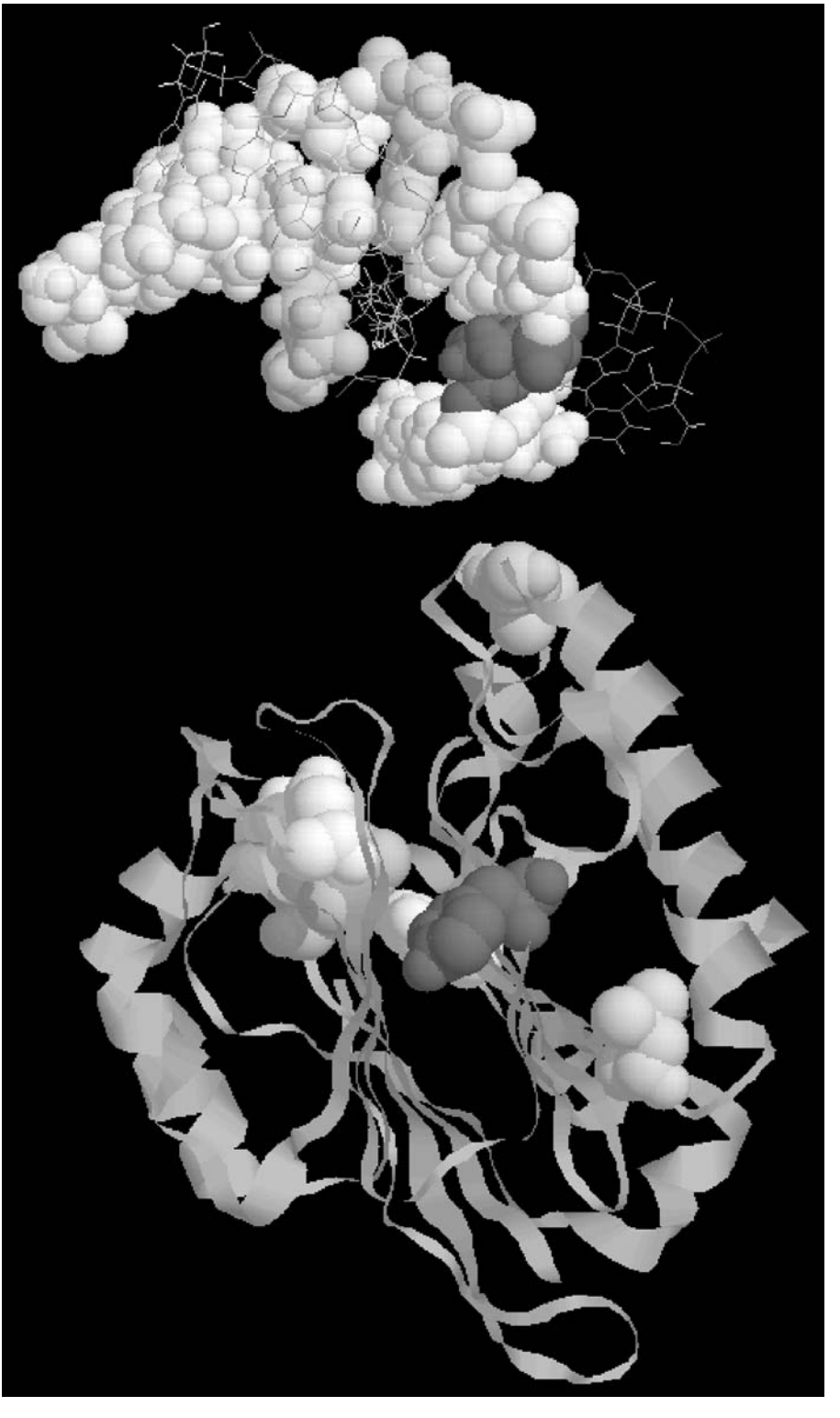
The human enzyme consists of 318 amino acids with a molecular mass of 35.5 kDa. The crystal structure of the enzyme lacking the first 43 N-terminal residues has been reported *(76)* and is shown in Fig. 2. The enzyme consists of two portions, the nonordered amino terminus that extends to amino acid 36 and is missing in the enzyme from prokaryotes, and an ordered globular domain comprising the remainder of the molecule *(231).* The unconserved first 36 amino acids include 11 positively charged residues, 8 negatively charged residues, and 4 proline and glycine residues that discourage the formation of alpha helices. The function of the amino terminus for DNA repair is unknown, because its removal does not appear to alter the ability of the enzyme to nick DNA *(7,95).* However, the amino terminus contains two potential nuclear localization sequences, one at residue 2 (PKRGKKG) and one at residue 21 (PEAKKSK) *(85)* and a potential mitochondrial transit peptide sequence at residue 14 *(173).* Although the function of the N-terminus in the nicking reaction is unclear, this portion of the protein is apparently involved in the redox activity described in Subheading 2.2.1.

The major enzymatic activity of AP endo is cleavage of the abasic site on the 5′ side of the phosphodeoxyribose in ds DNA *(58,82,231).* This activity requires a divalent cation with Mg^{2+} being preferred. Mn^{2+} will not substitute, although in some site-directed mutants, Ca^{2+} restores the activity to that of the wild-type enzyme *(151).* The nicking activity requires residues from amino acid 62 through the carboxyl terminus *(95).* Deletion mutants beyond NΔ61 are unable to nick abasic site-containing oligonucleotides nor provide resistance to methyl methane sulfonate in *E. coli* xth^{-} nfo^{-} double mutants *(95).*

Other enzymatic activities found in Exo III are markedly diminished in the human enzyme. These include a 3′ exonuclease activity, 3′ dRPase activity, 3′ phosphodiesterase activity *(34,50,58,234),* and an RNase H activity *(7,209).* Although the turnover number for the 3′ phosphodiesterase is 0.05 s^{-1} *(266)* and its activity is present at ~1% of the nicking activity, a strong case can be made for its importance under oxidative-stress conditions. The level of AP endo expression is increased when cells are exposed to nontoxic levels of a variety of ROS *(204)* that generate the need for the 3′ end removing activity. Izumi et al. *(93)* point out that the level of expression of AP endo remains unchanged by treatment with alkylating agents or ultraviolet (UV) light, where there is apparently sufficient number of molecules to perform the standard nicking reaction. In the case of oxidative stress, the 3′ phosphodiesterase activity may be rate-limiting. Although the protein is abundant, the necessary activity is low and the amount of enzyme is insufficient to meet the enhanced need.

Detailed kinetic analysis of the AP endo DNA strand scission reaction was accomplished using single-turnover kinetics. In this type of analysis, the enzyme undergoes one round of catalysis. When it dissociates from either substrate or product, it is prevented from undergoing a second catalytic round. Single-turnover analysis was made possible when it was demonstrated that the β-elimination product of a ds oligonucleotide containing an abasic site (HDP) acts as a powerful inhibitor of the nicking activity of the enzyme *(230).* In order to perform single-turnover studies, enzyme was allowed to bind to substrate in the absence of divalent cation. One round of catalysis by enzyme already bound to substrate was initiated by the addition of Mg^{2+} in the presence of HDP + heparin.

These studies made it possible to determine the kinetic binding and dissociation constants for substrate of the wild-type and several mutant enzymes and to demonstrate that the kinetic scheme followed by the wild-type enzyme is best described by a Briggs-



Haldane mechanism *(230).* The turnover number for the wild-type enzyme is 10 s^{-1}, whereas the catalytic efficiency is 1.0×10^8 $M^{-1}s^{-1}$ and the K_d is 0.8 nM. These constants serve as reference points for all subsequent studies.

AP endo has another activity that is ostensibly unrelated to ASR and that resides in the N-terminal portion of the molecule. The enzyme is able to promote DNA binding by p53 *(96)* as well as by the AP-1 transcription factors, Fos/Jun heterodimers or Jun/Jun homodimers, in vitro, in the absence of reducing agents. Binding was demonstrated by electromobility shift assay (EMSA) *(210A,270,271).* Activity for stimulating DNA-binding of Fos/Jun dimers as well as NFκB, Myb, AP-1 proteins, members of ATF/CREB family, and HIFα (hypoxia inducible factor) *(87,90,258,262)* is reported to require Cys^{65} because site-directed mutagenesis of AP endo Cys^{65} to alanine results in loss of the redox activity. Initially, Cys^{65} was hypothesized to form a disulfide bridge with Cys^{93}, which together could provide the oxidative function. Although these residues were initially conceived as being readily accessible to the surface of the enzyme *(254),* both domain-mapping and X-ray crystallography demonstrated that they are not *(76,231).* Because there are no disulfide bridges in AP endo, at least none stable enough to be found in the crystal structure *(76),* the two residues themselves may not be involved in redox activity; rather, they might maintain a conformation that allows other residues that are surface accessible such as the amino terminus to perform this function *(96).*

Although physical evidence for redox function of intact AP endo is not in hand, nuclear magnetic resonance (NMR) studies reveal that when a peptide comprising residues 59–71 of AP endo is mixed with human thioredoxin, the AP endo peptide binds in a crescent-shaped groove on the surface of thioredoxin *(201).* One study indicates that AP endo may be involved in regulating gene expression in response to hypoxic conditions *(261).* Smooth-muscle cells respond to oxygen deprivation by inducing the expression of heme oxygenase (HO-1) *(277).* In response to hypoxic conditions, thioredoxin becomes localized in the nucleus. Thioredoxin treatment of cells co-transfected with AP endo and the enhancer region for HO-1 enhanced gene expression of HO-1 threefold.

As stated earlier, in the crystal structure lacking the first 43 N-terminal residues, the enzyme is globular. The globular portion consists of two six-stranded β-sheets surrounded by α-helices, which together form a four-layered α/β sandwich arranged in a nuclease fold, a feature characteristic of many endonucleases. The cocrystal reveals that the enzyme inserts loops into both major and minor grooves, binding the flipped out AP

Fig. 2. Three-dimensional structure of human AP endonuclease *(76)* and a 12-mer oligonucleotide containing a single abasic site *(11).* Although abasic site-containing DNA does not fit spatially into the crystal structure, which was obtained in the absence of DNA, the oligonucleotide is shown to provide a sense of scale and to illustrate results described in the text. Active-site residues of AP endo are His^{309} (yellow), Asp^{283} (white), Asp^{308} (white), and Glu^{96} (white). Residues identified by domain mapping *(217)* as protease accessible but blocked in the presence of substrate/product are Leu^{179} (green), Tyr^{144} (red), and Leu^{205} (blue). The structure of the 12-mer with the single abasic site, obtained by NMR *(11),* is aligned to permit the viewer to visualized potential interactions with the active site and the groove along which the blocked sites lie. The abasic site is indicated as green space-filled atoms in a wireframe chain. The complementary strand is shown as space-filled, where the position opposite the abasic site is blue, the position located at -1 is identified in yellow and the position located at -3 is shown in red.

site in a pocket. The orphan base across from the flipped out AP site hydrogen bonds with the base 5′ to the AP site, which results in a kink in the DNA *(163a).* The active site is proposed to include His^{309} flanked spatially by two aspartate residues, Asp^{283} and Asp^{308} (Fig. 2). The importance of these residues for catalysis and substrate binding has been confirmed kinetically *(141)* and by EMSA *(150).* Kinetic binding studies provide the precise binding constants that are related to enzyme function, i.e., enzymatic cleavage, whereas EMSA studies provide data on structural binding of the enzyme to its substrate independent of enzymatic activity. Differences in results obtained by the two protocols occur frequently (176A, 210A, Fattal and Strauss, unpublished data) and may reflect sensitivity of EMSA to forces imposed by an electrical field as well as shear force from the supporting gel during electrophoresis, or may reflect a two-step binding process involving conformational change and/or the destabilization that occurs during electrophoresis. Asp^{283} and Asp^{308} are apparently involved in maintaining the spatial conformation of the active site histidine *(141)* and in providing a negatively charged environment that permits interaction with the abasic site. Kinetic studies have shown that mutation of either active-site aspartate diminishes binding of the abasic-site substrate to the enzyme by two orders of magnitude and enhances dissociation. Mutation of both active-site aspartates diminishes kinetic binding by four orders of magnitude *(141).* Mutation of His^{309} to asparagine decreased k_{cat} by 30,000-fold, while attempts to measure binding under single-turnover conditions were unsuccessful owing to weak enzyme-substrate interaction. Despite the striking differences in enzymatic cleavage and kinetic binding, the two aspartate mutations, alone or together, and the histidine mutant had near wild-type binding behavior on EMSA analysis *(150).* Other site-directed mutagenesis studies have also shown that residues required for shifts in electrophoretic mobility are not necessarily the ones required for nicking *(8,150).* These data argue that at least some of the residues involved in AP endo complex stability under EMSA conditions are not the same as those involved in catalysis.

In addition to kinetic and EMSA studies, molecular modeling indicates that replacement of either aspartate with alanine probably results in movement of other key residues in the neighborhood of the active site. Many of the residues that move and the degree of shifting are different for the two aspartates, again implying that the two residues are not strictly equivalent *(141).* Apparently these small, computer-predicted changes in conformation may result in large changes in enzymatic behavior but do not affect the behavior of the enzyme during gel-shift assays.

A number of other mutations at highly conserved residues have been prepared and examined for nicking activity under steady-state enzymatic conditions or for complementation by phenotypic screening in *Escherichia coli.* These include Glu^{96} *(7,8,9,94),* Asp^{70}, Asp^{90} Arg^{177} *(210a),* Asp^{210} *(61,210a),* Asp^{219}, Met^{270}(163A), Met^{271} *(163a),* Asp^{308} and His^{255} *(8),* Asn^{212} *(211),* Glu^{96}/Lys^{98} *(94),* Phe^{266} and Trp^{267} *(60),* and Tyr^{171} *(61).* Although all of the residues examined except Asp^{70} showed some deficiency in nicking, none show the deficiency demonstrated by conversion of His^{309} to alanine described earlier. Although Glu^{96} is essential for complementation in *E. coli,* the E96A mutation is suppressed by conversion of Lys^{98} to arginine *(94).* Finally, cleavage is enhanced in the R177A mutant *(163a).* In addition, AP endo variants that have been identified in the human population include L104R, E126D, R237A, D283G, D148E, G306A, and G241R *(80a).* The first three variants exhibited 40–60% reduction in incision activity, while the

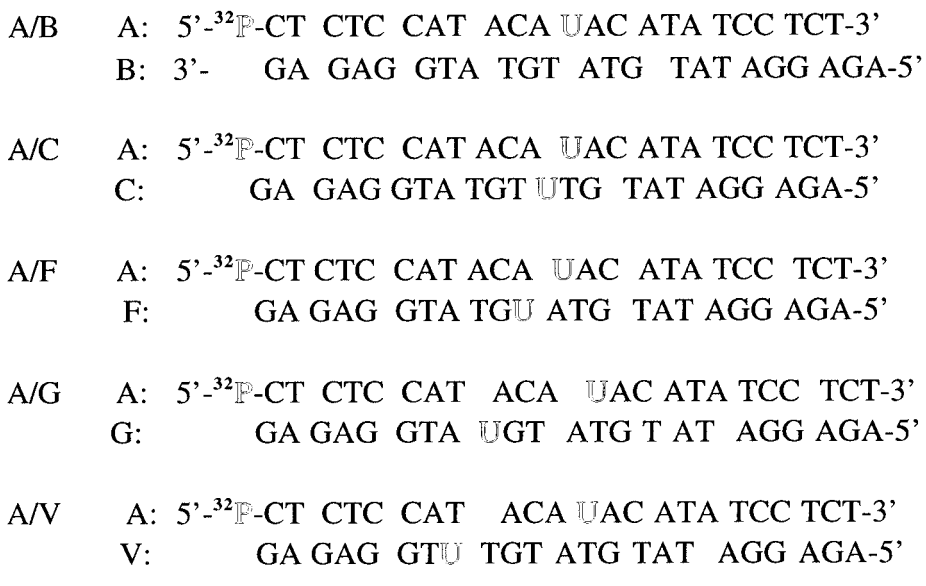

A/B
A: 5'-32P-CT CTC CAT ACA UAC ATA TCC TCT-3'
B: 3'- GA GAG GTA TGT ATG TAT AGG AGA-5'
A/C
A: 5'-32P-CT CTC CAT ACA UAC ATA TCC TCT-3'
C: GA GAG GTA TGT UTG TAT AGG AGA-5'
A/F
A: 5'-32P-CT CTC CAT ACA UAC ATA TCC TCT-3'
F: GA GAG GTA TGU ATG TAT AGG AGA-5'
A/G
A: 5'-32P-CT CTC CAT ACA UAC ATA TCC TCT-3'
G: GA GAG GTA UGT ATG T AT AGG AGA-5'
A/V
A: 5'-32P-CT CTC CAT ACA UAC ATA TCC TCT-3'
V: GA GAG GTU TGT ATG TAT AGG AGA-5'

Fig. 3. Bistranded abasic site-containing substrates.

fourth, D283G, behaved similarly to D283A *(141,150).* Although neither D14E nor G306A affected cleavage activity, G241R had slightly *increased* activity.

On the basis of the crystal structure of AP endo and by analogy with other nucleases where a co-crystal has been obtained, Gorman et al. *(76)* have proposed a mechanism by which AP endo might bind to and nick an abasic site (*see* Fig. 2). The human enzyme requires at least 4 bp 5′ and at least 3 bp 3′ of an abasic site for incision and makes contacts within both the minor and major groove and with both strands of DNA around the abasic site *(262,263).* Five residues interact with the phosphate that precedes the one in the abasic site; another six residues interact with the phosphate in the dRP site. An aromatic residue such as Phe^{266} or Trp^{212} might interact with the abasic site itself. Note that Erzberger et al. *(60,61)* report that F266A and W267A retain considerable activity, while the nicking ability of Y171A, which is not remarked upon by Gorman et al., is greatly reduced. In the mechanism proposed by Gorman et al. *(76),* Asp^{283} extracts a proton from $His^{309,}$ which then hydrogen-bonds with water. The oxygen from the water molecule then attacks the phosphate on the 5′ side of the abasic site, which was made more electropositive because of the polarization of the metal ion complexed to $Glu^{96.}$ This mechanism implies that substitution of Asp^{283} with alanine should leave a catalytically inactive protein, which it does not *(141,151).* However, mutagenesis studies indicate that *either* Asp^{283} or Asp^{308} might extract the proton from $His^{309.}$

AP endo binds specifically around the abasic site in DNA and causes a pronounced distortion at the abasic site in a preincision complex *(263).* In light of this observation, what is the minimal DNA structure that AP endo requires in order to recognize an abasic site? Because the enzyme acts efficiently on substrate where the abasic site has been reduced with $NaBH_4$ or replaced by tetrahydrofuran, the mutarotation that a deoxyribose is expected to undergo is not necessary for enzymatic cleavage *(263).* In addition, the enzyme maintains considerable activity when the abasic site is replaced by propane-

diol. No activity is retained when the abasic site is replaced by 2-(aminobutyl)-1,3-propanediol, implying that the DNA backbone is critical but that the remainder of the deoxyribose is not. However, positioning a phosphorothioate ester immediately 5′ to the abasic site was highly inhibitory, with the Sp isomer being far more inhibitory (>10,000-fold) than the Rp isomer (~20-fold). Hence, the molecular spacing from the nucleotide preceding the abasic site to the one following the abasic site needs to be maintained, as does the electronic configuration around the 5′ phosphate. Furthermore, enzymatic cleavage requires at least 4 bp 5′ to an abasic site *(263)*

The configuration of the DNA just upstream from the abasic site is of particular importance, as shown by the effects that a second abasic site has on cleavage of the first *(32,33)* (Fig. 3). If the second abasic site is located downstream to the abasic site in question and on the opposite strand, it seems to have little effect on nicking the first abasic site. On the other hand, if the second abasic site is located upstream and on the opposite strand as the first abasic site (for example, A/V in Fig. 3), it can have a major impact on the ability of the enzyme to nick the first abasic site. In interpreting the data, it is not possible to distinguish whether the presence of the second abasic site introduces a kink in the substrate such that it can no longer bind nor position the first abasic site for cleavage or whether the base opposite the second abasic site is able to "swing out" of the helix and interrupt binding of the enzyme in a position to cleave the first site. Physical studies of oligonucleotides with more than a single abasic site examined the impact of two tetrahydrofuran residues opposite each other *(73)* or displaced by one base in either the 5′ or the 3′ directing. *(130a)* When the abasic sites are opposite each other, the lesion has minimal impact on the duplex transition enthalpy, but decreases the melting temperature (T_m) by 12°C. Although the data could have been interpreted as leaving the sugar phosphate backbone essentially unchanged with solvent providing the hydrogen bonding between the two strands, the authors preferred to interpret their data in the following fashion: the two tetrahydrofuranylphosphate moieties rotate out away from the helix, thereby allowing the 5′ and 3′ flanking base pairs to collapse on one another to form a stack similar to that seen within the control duplex. When two abasic sites are located one on each strand in a 5′ or 3′ orientation to each other, the thermal stability of the duplexes is severely reduced, especially in the 5′ orientation. In the 5′ orientation the abasic sites take up an extrahelical location, which contrasts with their smooth alignment along the sugar-phosphate backbone in the 3′ orientation *(130a).* The one thing that is clear is that conformation of the DNA helix for about one half of a helical turn upstream of the abasic site is important for recognition and cleavage.

The aforementioned data become even more relevant in the context of whether enzyme and/or substrate might undergo conformational changes during binding and cleavage. In domain-mapping experiments, substrate with an abasic site bound to the enzyme slows proteolysis at three chymotrypsin-cleavage sites *(231).* To conduct these experiments a divalent cation was required in order for proteolysis to proceed. In order to perform these experiments, both substrate and enzyme had to be present in high, stoichiometric amounts. Hence, there was a mixture of both substrate and product by the end of the proteolytic period. If product binds to AP endo differently than substrate, as is likely from the EMSA studies presented earlier, then a mixture of structures will become apparent during the domain mapping. The three blocked sites visualized during domain mapping form a spherical triangle on the surface of the molecule on one side of

the nuclease fold (Fig. 2). The distances correspond to approximately one turn of a DNA helix for the first two blocked pairs and one half a turn of the helix for the third pair. Given the distances between the three residues and their steric relationships, it is unlikely that a single oligonucleotide could block all three sites simultaneously. We have proposed that the disturbance in the structure upstream from the abasic site is relieved when the enzyme nicks the abasic site. Hence, substrate and product probably bind differently to the enzyme and almost certainly undergo a conformational change as nicking proceeds. Despite the fact that the enzyme in the presence or absence of substrate or product shows little difference in tertiary structure *(163a),* we cannot exclude the possibility that it undergoes a conformational change as it executes the nicking reaction, so that if substrate and product could bind simultaneously to the same molecule, they would abut and block the three nucleotide residues upstream from the abasic site.

Finally the question arises as to how AP endo locates an abasic site in a long DNA molecule. Many DNA metabolizing enzymes do not dissociate from DNA between successive rounds of catalysis, whether the reaction is insertion of nucleotides, e.g., a polymerase, or cleavage, e.g., a nuclease *(79)* or a DNA glycosylase *(13).* Such enzymes are described as processive in contrast to distributive enzymes that dissociate after each catalytic round *(112).* Processive enzymes can remain associated with substrate for thousands of rounds of catalysis, as in the case of DNA polymerases involved in replication; for 100–200 nucleotides as in the case of UDG or *Eco R1,* or for 4–6 nucleotides as in the case of pol β. AP endo falls into the second category and remains associated with DNA for a minimum of 175–200 nucleotides before dissociating from a multi-abasic site containing concatemer. In short, AP endo, like UDG and *EcoR1,* is processive *(28).*

AP endo not only scans DNA for abasic sites, but it can also displace glycosylases that dissociate slowly from their product *(257).* For instance, in the case of thymine DNA glycosylase, the dissociation rate (k_{off}) of the glycosylase is so slow that on a substrate with a G/T mismatch, the half-life of the complex is 5–10 h. The presence of AP endo reduces the half-time to 30–60 min. Although physical evidence of interaction between AP endo and any of the glycosylases remains elusive, these kinetic data provide support for direct interactions of this DNA glycosylase and AP endo.

Very little is known regarding post-translational regulation of AP endo. AP endo has potential phosphorylation sites for at least six protein kinases including casein kinase II, casein kinase I, and protein kinase C(PKC). Indeed, AP endo is subject to phosphorylation by all three of these kinases to varying extents *(276)* (*see* Fig. 4A, lanes 1 and 2). However, only phosphorylation owing to casein kinase II is reported to be associated with loss of nicking activity *(263).*

Because AP endo is subject to phosphorylation by a variety of protein kinases, there has been speculation that it might also be phosphorylated by DNA-dependent protein kinase (DNA-PK). DNA-PK is involved in repair of damage from ionizing radiation and double-strand break repair. It is also involved in V(D)J recombination that occurs during lymphoblast differentiation. It requires the ends of double-stranded DNA, although in the test tube DNA constructs containing single-strand to double-stranded transitions also activate DNA-PK in vitro *(30,164).* Would DNA containing an abasic site activate DNA-PK and would an activated DNA-PK recognize and phosphorylate AP endo? Alternatively, would AP endo in the presence of its substrate be more or less susceptible to phosphorylation? Figure 4B, lanes 3–5, shows the activation and phos-

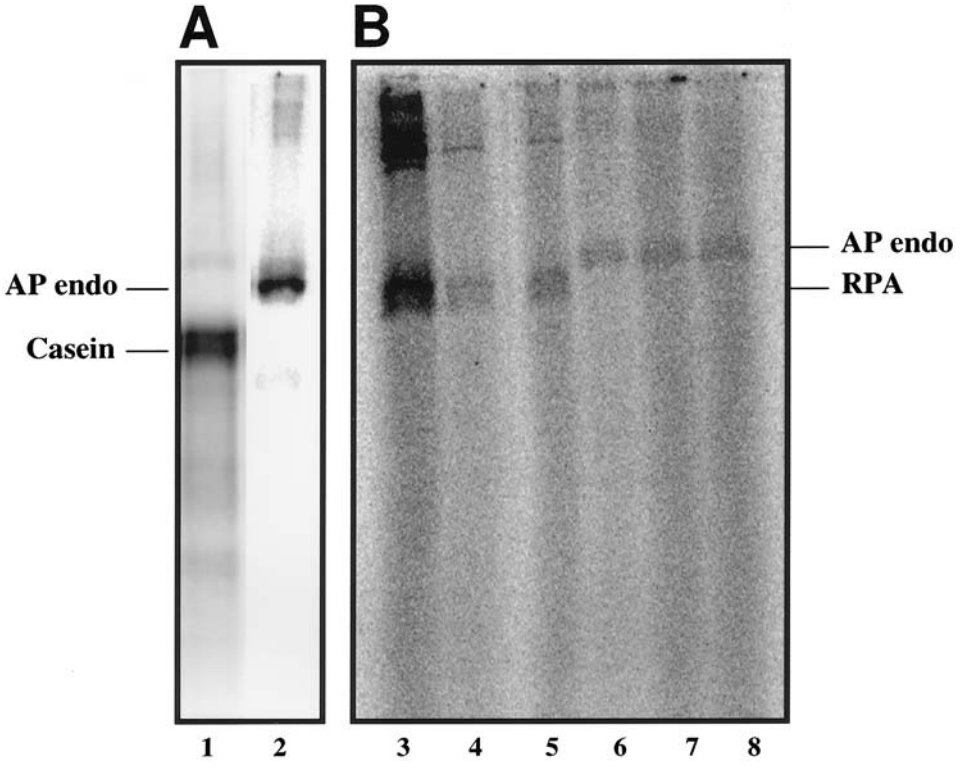


Fig. 4. AP endo is a substrate for casein kinase II but not for DNA protein kinase (DNA-PK). **(A)** Casein (12 pmoles) (lane 1) or AP endo (19 pmoles) (lane 2) was phosphorylated by casein kinase (500 U) as described by Yacoub et al. (276) and resolved by SDS-PAGE (12.5% gel). **(B)** RPA (72 pmoles)(lanes 3–5) or AP endo (36 pmoles)(lanes 6–8) was subjected to phosphorylation by DNA-PK (88 ng) as described by Chan and Lees-Miller *(30).* In lanes 3 and 6, the kinase was activated by 5 μg/mL sonicated calf thymus DNA; in lanes 4 and 7 the kinase was activated by the same amount of 45-mer oligonucleotide containing a U at position 21; in lanes 5 and 8 the kinase was activated by the same amount of 45-mer oligonucleotide in which the U had been removed to create an abasic site by means of uracil DNA glycosylase. Reaction volumes were 25 μL for casein kinase and 20 μL for DNA-PK.

phorylation of RPA, the traditional substrate for DNA-PK (*see* section on RPA under Auxiliary Proteins). Although sonicated calf thymus DNA activates the kinase towards RPA, oligonucleotide with or without an abasic site is unable to stimulate phosphorylation of RPA. When the protein to be phosphorylated is AP endo (Fig. 4B, lanes 6–8), poor phosphorylation is seen with all three sources of DNA. Thus, we conclude that AP endo is not a good target protein for DNA-PK.

AP endo was once thought of strictly as a housekeeping gene, because it is present in all cells and tissues. However, the level of expression varies with tissue type, developmental stage, degree of malignancy *(39,102,206,265,274),* Ca^{2+} concentration *(183),* and in the response of colon cancer cells to hypoxia *(275).* Furthermore, AP endo apparently interacts with p53, the tumor-suppressor gene most commonly associated with human cancer and that is responsible for cell-cycle arrest in G1 or G2 when cells are exposed to

γ-irradiation *(69,160,181).* Some groups report that expression of AP endo is activated in human and rodent cells by sublethal levels of ROS but not other genotoxic agents including alkylating agents *(93).* It is also activated by exposure to asbestos *(68).* The importance of AP endo in fetal development is clear from the fact that homozygous embryos do not survive beyond day E6.5 *(272).* As of this writing, a viable homozygous null cell line has not been established (B. Demple, personal communication)

2.2.2. The Polymerases

2.2.2.1. DNA Polymerase β

The Polymerase Activity The major DNA polymerase thought to be responsible for short-patch repair is DNA polymerase β (pol β) *(16,67,109,227).* Pol β is capable not only of DNA synthesis but also of 5′ -dRP lyase activity, as described in next paragraph. Not only has pol β no proofreading capability, but also it has the lowest fidelity of all the DNA polymerases *(119,120)* and may be responsible for a large part of the errors attributable to BER *(88).* Pol β shows maximal efficiency and maximal fidelity on 5′-phosphorylated 1-nucleotide gapped DNA *(31,189).* The average pol β error rate is ~6×10^{-4} *(119,120),* which is much higher than that of other DNA polymerases. Indeed, overexpression of pol β in CHO cells has been reported to give rise to a mutator phenotype with decreased sensitivity to cisplatin *(26).*

This 39 kDa protein has two major domains *(117,118):* the amino terminal 8 kDa (residues 1–75) and the carboxyl 31 kDa (residues 87–334) separated by a protease-sensitive hinge region *(190).* Pol β can use either Mg^{2+} or Mn^{2+}. The crystal structure is available in several different conformations with and without template primer and substrate dNTP *(189,190,220,221).* The current model for binding of pol β to a gapped DNA substrate requires a 90° bend in the single-stranded template, which probably enhances nucleotide selectivity during DNA synthesis. Thus, the series of X-ray crystallographic structures provides evidence for an induced-fit mechanism in terms of binding the incoming dNTP *(221).* Both domains are involved in recognition of substrate and catalysis as shown by cross-linking studies *(40,228),* and X-ray crystallography *(221).* The hinge region can influence fidelity, as shown by site-directed mutagenesis followed by fidelity studies *(185).* For example mutation of a single tyrosine in the hinge region results in an increase in both base substitution and frame-shift errors. Stopped-flow fluorescence studies reveal that there are multiple conformational changes during the catalytic cycle *(97,203,282).* Pre-steady state analysis has been used to determine theoretical levels of processivity for polymerization by pol β, which are low in comparison with other eukaryotic polymerases *(260).* Both chemistry and a conformational change are rate-limiting for nucleotide incorporation *(260).*

The 8 kDa domain contains the dRP lyase activity *(157,197).* A Schiff base is formed between Lys^{72} and the dRP residue. Mutation of this residue diminishes dRPase activity by ~90% while leaving DNA binding unaffected *(197).* Processive gap filling up to six nucleotides requires a 5′-phosphate group that is recognized by the 8 kDa domain *(199).* Pol β is processive in that it is capable of inserting up to six residues without dissociating from the template *(1,226).* In terms of fidelity, the accuracy of inserting one nucleotide into a single nucleotide gap exceeds fidelity of repairing other substrates *(31).* Although processive filling of a 5-nucleotide gap results in a similar base substitution fidelity as distributive synthesis along a longer template, closely spaced base sub-

stitutions are produced at a rate more than 60-fold higher than for distributive filling of gaps >300-nucleotides *(188)*. Whether the enzyme exhibits processivity in locating nearby gaps on the same DNA molecule, as might occur with damage spaced by nucleosomes, is not known at this time.

Site-directed mutagenesis has been used extensively to study structural and functional aspects of pol β enzymatic activity. Despite initial thoughts to the contrary, site-directed mutagenesis of several residues in pol β show that hydrogen bonding to the incoming dNTP or terminal primer nucleotide is *not* required for either high catalytic efficiency or nucleotide discrimination. Hydrogen bonding, however, is required between the enzyme and the template strand *(10,187)*. In other cases at least one mutation in the carboxyl end of the protein acts as a dominant negative and enhances mutation rates *(38)*.

The importance of pol β during embryogenesis is demonstrated by the fact that there are no viable homozygous knockout offspring and embryos die at midgestation day E10.5. Cells deficient in pol β through knockout mutation are hypersensitive to induction of apoptosis and chromosomal breakage by methylating agents and cross-linking antineoplastic drugs such as mitomycin C and mafosfamide *(180,227)*. They are not hypersensitive to agents that cause oxidative damage *(227)*. Pol β is inactivated by in vitro phosphorylation with PKC *(240)*. It is also a substrate for poly(ADP-ribosyl) polymerase (PARP) *(182)*. The physiological relevance of these observations is not understood at this time.

The Lyase Activity Evidence that the end-trimming or tailoring activity (lyase function) required to remove the 5′ dRP residue might be a function of pol β was first presented in 1995 *(155)*. Since that time, the classic imino intermediate found for all lyase endonucleases was demonstrated *(191)*. The lyase activity resides in the 8 kDa domain with Lys^{72} forming a Schiff base *(62,157,197,198)*. Conversion of Lys^{72} to alanine abolishes most of the activity. A preincised site is preferred, because k_{cat} for lyase activity on an intact abasic site is ~1/200 that of the site previously cleaved with AP endo *(198)*. Finally, lyase activity does not require divalent cation *(198)*.

Other proteins that also perform dRPase activity have been described, leading to speculation that pol β may not be the only protein to perform the step in ASR. For instance, both T4 DNA ligase *(19)* and *Drosophila* ribosomal protein S3 *(218)* have lyase activity. In mitochondria, which lack pol β, DNA polymerase γ (pol γ) performs the lyase function *(139,198)*. The physiologically relevant dRPase is not clear or perhaps this activity is redundant. Of particular interest is that FEN1 does NOT have an associated lyase activity *(46)*.

2.2.2.2. Replicative DNA Polymerases from the Nucleus

Two DNA polymerases involved in replication of nuclear DNA have been implicated in long-patch repair. They may also be involved in short-patch ASR when pol β is missing *(16,63,158)*. DNA polymerase δ (pol δ) and/or DNA polymerase ε (pol ε) can participate in long-patch BER in vitro. In some cases PCNA and RFC (Replication Factor C) but not RPA are required *(232)* but others report that RPA but not RFC is necessary *(47)*. Although both polymerases are capable of replacing a single nucleotide at the lesion site, the repair reaction is delayed compared to the rate of single nucleotide replacement by pol β.

Human pol δ is comprised of two subunits of 125 kDa and 50 kDa *(20).* Pol δ has 3′ to 5′ exo activity that can correct a terminal mismatched base. The error rate is $<1/10^6$ base insertions. Pol δ functions as a dimer in replication, where the structure is likely to coordinate the synthesis of leading and lagging strands. Its processivity depends on PCNA, which leads to a reduction in K_m and an increase in V_{max}. Thus, PCNA may enhance processivity by increasing both the residence time of pol δ on the DNA template-primer and the rate at which individual nucleotides are incorporated *(176).*

The second nuclear DNA polymerase that may be involved in long-patch ASR is pol ε, which is comprised of a 261 kDa catalytic subunit with a 55 kDa tightly associated peptide *(20).* The error frequency for synthesis by pol ε is 8×10^{-4} *(238).* Pol ε is probably involved in Okazaki fragment maturation, because it functions more efficiently as gap size decreases *(167)* and its processivity does not require PCNA at low-salt concentrations *(122,233,278).* Despite this fact, pol ε is able to drive PCNA/RFC dependent processive DNA synthesis under physiological salt concentrations (>0.1 *M* NaCl) *(126,149,278).* Pol ε has 3′ to 5′ exonuclease proofreading capability and binds ss DNA but not ds DNA *(126).*

2.2.2.3. Replicative DNA Polymerase from the Mitochondrion

The large quantities of reactive oxygen species generated by mitochondria mean that oxidative damage to mitochondrial DNA is a constant hazard for the cell (*see* Subheading 2.6.). The major polymerase involved in replicating mitochondrial DNA also plays an important part in protecting mitochondrial DNA from oxidative damage. Mitochondria have a unique DNA polymerase, DNA polymerase gamma (pol γ), and do not contain pol β, pol δ, or pol ε. Although the catalytic protein is comprised of a single 136 kDa subunit *(78,140)* with polymerase and 3′ to 5′ exonuclease activity *(140)* as well as the dRPase activity necessary for successful ASR *(139),* the polymerase functions as a dimer.

Replication by pol γ is by a strand-displacement mechanism involving mitochondrial single-stranded DNA-binding protein that stimulates processivity rather than stimulating primer recognition. Binding of pol γ to single-stranded (ss) DNA blocks action of its 3′ to 5′ exonuclease proofreading function. Careful analysis of catalytic steps in incorporation of dNTPs into mitochondrial DNA demonstrates a maximum polymerization rate of 3.5 s^{-1}, which could sustain replication of the mitochondrial genome in a physiologically relevant time-frame *(78).* Pol γ can be stimulated by PCNA *(236).*

2.2.3. Flap Endonuclease 1

Flap endonuclease 1 (FEN1), a 42 kDa protein *(86),* is a member of the structure-specific endonuclease family and repairs nicked double-stranded DNA substrates that have the 5′-end of the nick expanded into a single-stranded tail *(104).* FEN1 has both exonucleolytic and endonucleolytic activities. During lagging strand synthesis, it removes RNA primers from Okazaki fragments *(5,128),* leaving a nick or a single nucleotide gap. With a 5′ flap, FEN1 slides from the 5′ end of the DNA to the point of annealing to perform cleavage *(171).* It is able to cleave an unannealed flap with a dRP at the 5′ end but it has no dRpase activity *per se (46).*

FEN1 has been used to reconstitute long-patch ASR pathway by several groups *(105,109,134,158).* In particular, in a reconstituted system with proteins derived from *Xenopus laevis,* FEN1 was able to excise the 5′-incised abasic site in the PCNA-dependent pathway. DNA synthesis was not required for this activity if PCNA and a replica-

tion factor C-containing fraction were present. The pol β dependent pathway could also use FEN1 for excision of the synthetic abasic sites, which were not susceptible to β-elimination and which had been cleaved on the 5′ side by AP endo. In the latter case, PCNA and replication factor C were unnecessary but DNA synthesis was required *(71)*.

2.2.4. The Ligases

The two ligases known to participate in ASR are ligases I and III *(241,242)* and a newly discovered mitochondrial ligase *(181),* which is a variant of ligase III *(121)*. All ligases require ATP as a cofactor in order to join DNA *(135)*. The enzymes first form a covalent bond with AMP, transfer the AMP to the 5′ -phosphate terminus of the nick to be sealed in the DNA, and then seal the nick while they remove the AMP. The ligases are differentiated by structural elements, substrate range, gene locus, and subcellular location.

Ligase I is 125 kDa with an 85 kDa C-terminal catalytic domain. After binding to the site requiring ligation through the action of PCNA, ligase I is cleaved to generate the catalytically active C terminal fragment. In terms of synthetic substrates, ligase I ligates oligo dT•poly dA and oligo rA•poly dT, but not oligo dT•poly rA. In terms of physiological substrates, ligase I normally joins Okazaki fragments during DNA replication and is likely to be involved in strand breaks resulting from alkylating agents and ionizing irradiation. Ligase I interacts directly with pol β *(57,200)*. Furthermore, ligase I inhibits strand displacement of pol δ whether or not PCNA and RFC are present. Ligase I stimulates DNA pol ε in a PCNA-dependent manner *(166)*. Stimulation occurs when PCNA is present in low concentrations but at higher concentrations of PCNA, stimulation no longer occurs. DNA ligase I knockout mice are embryonic lethals at day E6.5 *(14)*

DNA ligase IIIa is a polypeptide of 922 amino acids that ligates all three synthetic substrates described earlier. The amino terminus is responsible for binding to the PCNA homotrimer, whereas the carboxyl end of the molecule is able to bind XRCC1 via BRCT motifs.

Mitochondrial ligase is a 100 kDa protein that forms an enzyme-adenylate intermediate consistent with the known mode of action of other ligases. This ligase, which can ligate oligo (dT) strands annealed to poly(rA), is a variant of ligase III *(121,192)*.

The genes for DNA ligases I and III have been cloned and the sites on the human chromosome have been identified. Human ligase I, LIG1, is ubiquitously expressed, although expression is highest in thymus with elevated levels in testis. Expression increases after UV irradiation. Human ligase III, LIG3, has two gene products differentiated by alternative splicing. The mRNA species encoding ligase III-α is ubiquitously expressed but the DNA ligase III-β mRNA only occurs in the testis. Consequently, the two may play distinct roles in germ-cell metabolism *(242)*. LIG3 also encodes mitochondrial ligase *(121)*.

2.3 The Auxiliary Proteins

2.3.1 Proliferating Cell Nuclear Antigen

PCNA was first discovered as an autoantigen in patients with lupus erythematosus and was later shown to be a component of both replication and repair *(100)*. PCNA is a sliding clamp composed of three 29 kDa subunits (258 amino acids each) that functions as a processivity device by clamping a DNA polymerase to DNA. The presence of PCNA increases processivity as much as 100-fold *(245)*.

The crystal structure shows a closed circular ring formed from tight association between three monomers *(111).* Each PCNA monomer is composed of two domains that fold to form a "quasi-six-fold symmetry" in the PCNA trimer. The trimer is the functional unit that acts as a clamp enabling DNA polymerases δ and ε to interact efficiently with DNA and proceed in a processive fashion. PCNA is itself assembled onto DNA by the clamp-loading replication factor (RFC; *see* Subheading 2.3.4.). PCNA interacts with a multiplicity of DNA associated proteins including but not limited to DNA polymerases δ and ε, clamp-loader replication factor (RFC), FEN1 *(128),* DNA ligase I *(127),* and $p21^{Cip1/Waf1}$ *(251),* as reviewed by Tsurimoto *(245).* The interaction with pol δ is via the small subunit *(285).* Site-directed mutagenesis has been used to identify the residues involved in binding many of these factors *(99).* In particular, a hydrophobic pocket is formed that is important for interacting with pol δ, p21, FEN1, and ligase I. In order for PCNA to stimulate the FEN1 activity, PCNA must be below the 5′ flap *(99).* The fact that PCNA is loaded onto DNA in a fixed orientation relative to the direction of DNA permits discrimination of newly synthesized DNA strands from parental strands during replication. It may also have a role in orienting the repair process, at least in mismatch repair *(245)* (*see* section on interactions with other DNA repair systems).

Not surprisingly, PCNA *promotes misincorporation* catalyzed by pol δ *(168)* by decreasing the off rate of the pol δ•template-primer complex, e.g., by increasing stability. In fact, it stimulates bypass synthesis by pol δ 53-fold past an abasic site *(169).* Incorporation is primarily dA in accordance with "A rule." $p21^{Cip1/Waf1}$, an inhibitor of PCNA-dependent DNA replication, inhibits PCNA-stimulated synthesis in vitro past model abasic template sites. (Y. Matsumoto, personal communication).

PCNA is involved in both nucleotide excision repair and the long-patch pathway of ASR. In particular, involvement with the latter pathway was shown by reconstitution with AP endo, RFC, PCNA, FEN1, pol δ, and DNA ligase I. Neither RPA nor Ku protein complex enhanced the repair activity in this system *(232).* Others have reconstituted long-patch BER with RPA instead of RFC *(47).* In a recent study using human cell extracts, RPA and PCNA complemented each other; neither was able to replace the other *(54).*

2.3.2. $p21^{Cip1/waf1}$

$p21^{Cip1/waf1}$ is a cyclin-dependent kinase inhibitor that binds to PCNA and subsequently modifies its activity. In fact, p21 and FEN1 bind in a mutually exclusive fashion to PCNA. When p21 is bound to PCNA, it prevents PCNA from participating in DNA replication and possible repair. The protein induces G1 arrest and can block the onset of S phase in response to DNA damage *(70).* It also inhibits repair of DNA by pol δ, DNA ligase, RFC, PCNA, and FEN 1 (Kim et al., personal communication). However, it fails to inhibit pol β-dependent repair of abasic sites. Both DNA synthesis and abasic-site excision by FEN1 are suppressed by p21 in vitro. Loading of PCNA on circular DNA containing an abasic site is also blocked by p21, but preloaded PCNA is relatively resistant to p21. Consequently, it appears that p21 suppresses PCNA-dependent ASR by blocking loading of PCNA on DNA *(106).*

2.3.3. Replication Protein A

RPA (replication protein A, also known as replication factor A or RFA) is a heterotrimer ss DNA binding protein *(267).* It is the most abundant of single-stranded

DNA binding proteins. hRPA comprises 3 subunits, 70, 32, and 14 kDa, known as hRPA70, hRPA32, hRPA14, respectively.

During replication, RPA is required for DNA synthesis reconstituted from purified proteins and is part of the large 17-S multiprotein complex that includes pol δ and/or ε. RPA binds ssDNA nonspecifically. When bound to ssDNA, which occurs during S phase and after DNA damage, the 32 kDa subunit is phosphorylated by DNA-PK. Phosphorylation occurs in a cell-cycle dependent fashion or in response to DNA damage induced by ionizing radiation or UV light *(21,29,137)* and leads to enhanced unwinding ability *(29,137).* The RPA·ssDNA complex is very stable (binding constant = 10^9) and can tolerate urea up to 6 *M* and guanidine.HCl up to 2 *M.* The two ssDNA binding domains reside in the RPA70 subunit, although the C terminus of hRPA70 is not itself involved in binding *(75).* The complex of hRPA32 and hRPA14 also has a single-strand DNA-binding domain *(18).* The protein prefers binding to pyrimidine tracts over purine tracts.

RPA stimulates completion of long-patch ASR *(47)* in a salt and PCNA-independent fashion, which means the stimulation is independent of its ability to unwind dsDNA. However, involvement in DNA repair may require recognition of single-stranded regions. RPA also stimulates BER in PCNA-dependent repair of abasic sites *(54).* Although the repair proteins with which RPA interacts are largely those required for damage recognition and excision in nucleotide excision repair, e.g., XPA, XPG, and ERCC-1/XPF, RPA also interacts with the RAD52 protein, which is itself essential for double-strand DNA-break repair and with XPG *(72).* The BER proteins with which RPA interacts include UDG *(170,172)* and FEN1 *(17),* which it stimulates. RPA is also ADP-ribosylated, the significance of which remains unclear at this time *(268).*

2.3.4. Replication Factor C

RFC is a five-subunit protein complex (p140,p40,p38,p37, and p36) that acts as a clamp loader for PCNA onto DNA. Either pol δ or pol ε then associates with DNA at the primer terminus, at which time RFC dissociates from the complex *(195).* ATP hydrolysis is required for loading. Human RFC has been reconstituted from its five subunits simultaneously expressed in baculovirus-infected cells *(22).* Apparently all five subunits constitute the ATPase activity required for loading *(23,194).*

The large subunit contains the DNA and PCNA binding domains *(65).* DNA primer-end recognition and PCNA binding activities are located in the C-terminal half of p140, whereas the N-terminal half of the protein is not required for RFC complex formation, replication activity, and PCNA loading *(246).* The large subunit is also a substrate for phosphorylation by Ca^{2+} calmodulin-dependent protein kinase II. RFC is inactive upon phosphorylation *(146).* RFC has been used as an important component in reconstituting long-patch BER when pol δ or pol ε are involved *(232).*

2.3.5. Poly(ADP-ribose) Polymerase

PARP is a 122 kDa protein with an amino terminal DNA-binding domain, a central automodification domain and a C-terminal catalytic domain. The enzyme ADP ribosylates nuclear proteins either in a straight chain or in a branched chain at the site of a DNA-strand break *(48,49,212).* The proteins that it targets are those involved in chromatin architecture and DNA metabolism or it can auto-ribosylate itself. PARP molecules carrying long chains of branched ADP-ribose polymers lose their affinity for DNA and become inactivated. Ribosylated PARP then loses its poly(ADP-ribose) through the action of poly(ADP-ribose)glycohydrolase, which degrades protein-bound polymers down to the

protein-proximal ADP-ribose residue. The first residue is removed by ADP-ribosyl protein lyase so that PARP is ready for a new round of nick-binding and automodification.

Two observations make it likely that PARP is involved in activating proteins involved in repair of single-strand breaks in DNA. First, nuclear proteins activated with PARP bind to single-strand DNA breaks, and, second, knockout mice homozygous for the PARP deletion are extremely sensitive to methyl nitrosourea (MNU) and to γ-irradiation. Cells from the knockout mice are highly sensitive to methylmethane sulfonate, which causes growth retardation, G2/M arrest, and chromosome instability. Although they are delayed in DNA break resealing, cell viability is restored after transient expression of the PARP gene. PARP ribosylates pol β *(182)* and binds XRCC1. Thus, PARP is likely to play an important role in activating ASR in vivo *(244).*

2.3.6. XRCC1

X-ray Cross Complementation Protein 1 (XRCC1) is a 70 kDa protein with 633 amino acids *(239)* whose function is best described as a "nick sensor" *(24).* The protein binds with pol β, ligase III, and PARP. Knockout mice for XRCC1 do not survive embryogenesis. XRCC1 is detected in all tissues but is most prevalent in testis *(284).* XRCC1 functions as a homodimer, interacting via a BRCT domain in the C terminus. The BRCT site provides the site for interaction with ligase III as determined by far Western and affinity-precipitation analyses *(174,281).* The solution structure of the N-terminal domain is now available, revealing that the terminal domain binds both gapped and nicked single-stranded DNA and a gapped DNA-pol β complex *(148).*

The gene for XRCC1 complements a defect in CHO mutant EM9 cells, which are 10-fold more sensitive to ethylmethanesulfonate and ~twofold more sensitive to ionizing radiation. These cells have reduced ability to rejoin single-strand DNA breaks and a 10-fold elevated level of sister chromatid exchange compared with the CHO parental line *(239);* EM9 is also hypersensitive to camptothecin, an inhibitor of topoisomerase I. The last effect is independent of DNA replication *(6).*

In vitro studies have shown that XRCC1 interacts with one of the two forms of DNA ligase III *(25,174),* with pol β and possibly with PARP *(24).* The interaction with PARP, which was demonstrated by the yeast two-hybrid system, occurs via the central region (amino acids 301–402), which contains a BRCA1 C-terminus. Overexpression of XRCC1 in COS cells decreases PARP activity in vivo *(149).*

The CHO EM-C11 cell line has greatly reduced levels of XRCC1. Extracts from these cells are partially defective in ligation of BER repair patches in comparison to WT CHO-9 extracts. Treatment of CHO EM-C11 with alkylating agents results in an altered spectrum of mutations in comparison with parent CHO-9 line, consistent with the hypothesis that reduced ligation efficiency of single-strand breaks generated during ASR owing to XRCC1 levels may lead to deletions *(186)* When added to an in vitro BER system, XRCC1 suppresses strand displacement by pol β *(116),* an observation that suggests that strand displacement is not the optimal repair mechanism, provided that pol β is available for repair synthesis.

2.4. Interactions Between Different Components of the ASR Pathway

That the different components in the BER pathways must recruit or interact with one another is intuitively obvious but experimentally difficult to demonstrate. The most compelling evidence for recruitment is provided by Fortini et al. *(64),* where the selec-

tion of the repair route depends on the initial lesion, implying that the first enzyme to recognize the damage controls the selection of the entire pathway. Other clues to interactions include data showing that in the yeast two-hybrid system pol β interacts with AP endo. Furthermore, the two proteins cause an EMSA supershift when the substrate is abasic site-containing DNA *(12).* Prasad et al. *(200)* demonstrated that pol β and ligase I not only co-immunoprecipitate but also migrate together on glycerol gradient centrifugation analysis. Waters et al. *(244)* also demonstrated AP endo can enhance the turnover of several glycosylases. XRCC1 has binding sites for PARP, ligase III, and pol β, leading to speculation that XRCC1 is a scaffolding on which ASR takes place *(148).*

On the other hand, some authors feel that the nature of the gap and the form and location of the abasic-site deoxyribose determine which repair pathway occurs. Short-patch repair is the mode of choice because AP endo and pol β are such efficient enzymes. However, if the 5′-deoxyribose has been reduced or altered so that pol β cannot cleave it from the downstream strand, if the 5′-deoxyribose is missing as occurs with a gap or if both the 5′-deoxyribose and the next 5′-phosphate are missing, then long-patch repair is more likely to occur. Because long-patch repair requires the presence of PCNA, any substrate that cannot retain PCNA, e.g., a relatively short oligonucleotide, will not undergo long-patch repair. Consequently, so long as the substrate is a short oligonucleotide, BER will proceed poorly by either mechanism in extracts from pol β –/– cells. This line of thought does not take into account those observations where the pathway appears to be determined largely by the initial lesion.

2.5. Molecular Interactions with other DNA Repair Systems

Recently, a number of findings have demonstrated that in humans, ASR and other major DNA-repair systems of the cell share many of their molecular components (*see* Fig. 5). Experimental data suggest that an essential nuclease in nucleotide excision repair (NER) plays a role in ASR of at least one form of oxidative damage. In addition a protein complex, central to the mismatch repair process in human cells, also acts to couple the excision by BER of an oxidative lesion to transcription. PCNA and the polymerases δ and ε are essential parts of the DNA replication machinery and are shared among several of the major repair systems, while AP endo and PARP may have overlapping roles in both ASR and double-strand break repair (DSBR).

2.5.1. XPG Plays a Role in NER and BER

NER is the pathway by which several helix-distorting lesions, including those caused by UV light and certain carcinogens, are removed from DNA. It is a complex multiprotein process involving dual incision on either side of the lesion to be excised and subsequent removal of the oligonucleotide containing the damage *(215).* Two different structure-specific nucleases are used to create the dual incision. In mammalian cells, these are the XPG protein, which makes the 3′ incision, and the ERCC1-XPF complex, which makes the 5′ incision *(268).*

Defects in NER are generally nonlethal but mutations in any of the seven genes involved in the early steps of the process (XPA through XPG) result in the hereditary disease xeroderma pigmentosum (XP). XP patients exhibit acute sun sensitivity, marked skin changes in exposed areas, susceptibility to skin cancer, and frequently progressive neurological degeneration.

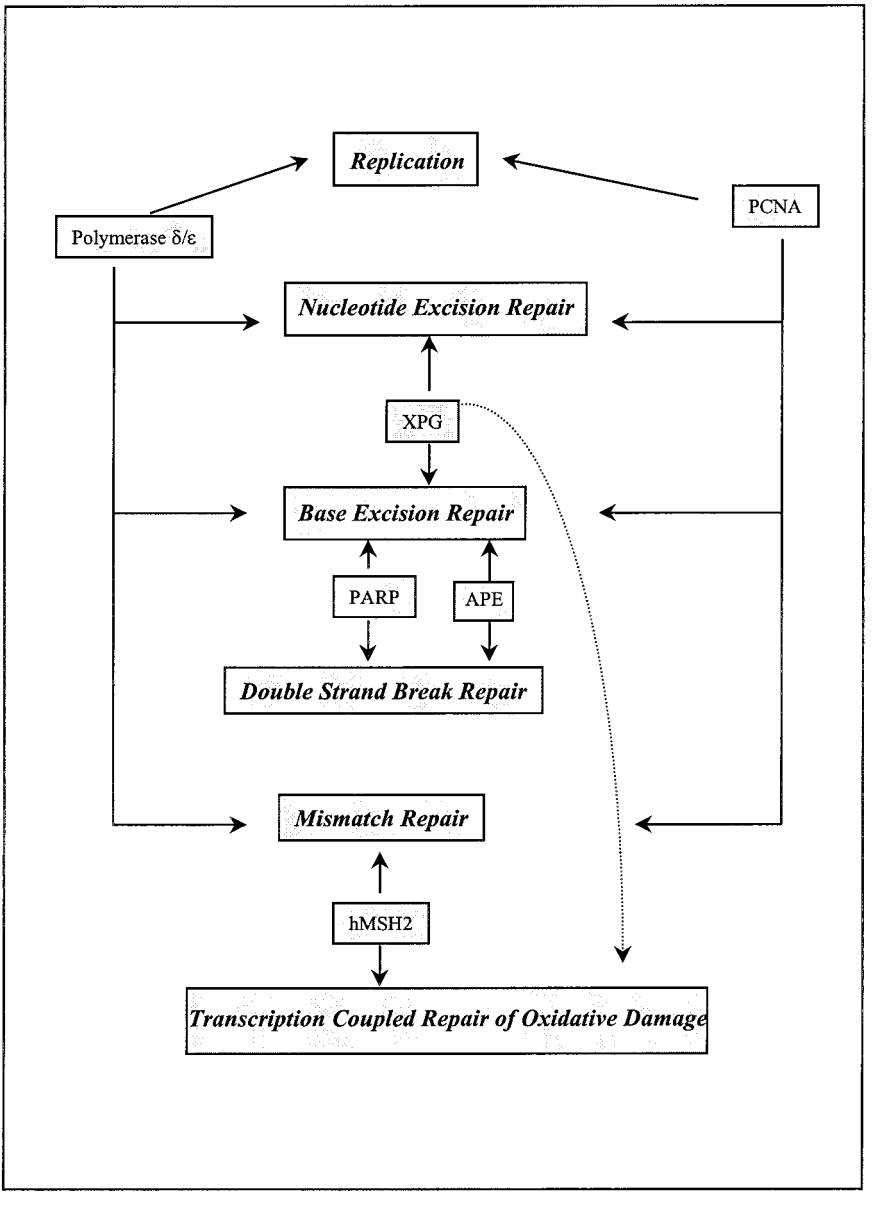


Fig. 5. Abasic site repair shares molecular partners with other DNA repair and replication systems. PCNA and polymerase δ/ε are essential components of replication and are shared among three of the major DNA repair systems including mismatch repair, base excision repair, and nucleotide excision repair. XPG is an essential nuclease of nucleotide excision repair and in addition plays a role in the global and transcription-coupled repair of oxidative damage facilitated by the enzymes of base excision repair. HMSH2, a required component of mismatch repair, may also help couple the repair of oxidative lesions to transcription. PARP and AP endo may play a role in both base excision repair and double-strand break repair.

Cockayne's syndrome (CS) is also a rare human photosensitive disease with a recessive inheritance pattern. CS patients suffer from developmental and neurological abnormalities. Death results from progressive neurological degradation before the age of 20 years. CS patients display an increased photosensitivity of the skin, but unlike XP patients, do not develop skin tumors *(248).* Cells from patients with CS are defective in the preferential removal of lesions from the transcribed strands of active genes by a transcription-coupled repair (TCR) process, including removal of specific lesions targeted by ASR, as discussed later. Classic CS is caused by mutations in either the CSA or CSB genes *(243).* CS and XP are usually clinically and genetically different but complementation studies have assigned some CS patients to the XP groups B, D, or G *(89,249).* Recently it was demonstrated that three CS patients had mutations in XPG that would produce severely truncated protein. In contrast, two sibling XPG patients without CS were found to have a missense mutation in XPG that inactivated its function in NER but produced full-length protein *(179).* Such findings have led to the conclusion that the clinical presentation of CS in patients with XPG mutations is not related to the incision function of XPG in NER but entails a second function for the enzyme that requires full length protein.

The NER/Cockayne's connection has now been extended to BER. Oxidatively damaged bases such as thymine glycol (Tg) are among the most abundant lesions resulting from ionizing radiation and other processes that generate reactive oxygen species *(256).* The removal of Tg lesions from DNA by the BER pathway is initiated by hNth1, a bifunctional enzyme that acts both as a DNA glycosylase to remove the altered base and also as an AP lyase by cleaving the DNA backbone. CS patients from the XP complementation group G who produce severely truncated XPG protein exhibit a reduced ability to remove Tg from their DNA after exposure to ionizing radiation *(41).* This observation has led to the hypothesis that certain domains of XPG may be essential for removal of Tg lesions in DNA.

The repair of oxidatively damaged DNA through BER has been reconstituted using purified human proteins, hNth1, AP endo, pol β, and DNA ligase III-XRCC1 in conjunction with substrates containing oxidized forms of pyrimidines, i.e., Tg and dihydrouracil *(110).* The initial step of the reaction was found to be strongly stimulated by purified human XPG, which promotes binding of hNth1 to oligonucleotide substrates containing damaged DNA. XPG proteins carrying mutations that disable nuclease function but yield full-length protein were found to stimulate hNth1 activity in a similar fashion to wild-type enzyme. These findings suggest that XPG activates the BER of oxidative DNA damage by promoting the binding of hNth1 to its DNA target and this activation is independent of its role as a nuclease in NER. Development of CS in XP-G patients may therefore be related to inefficient removal of endogenous oxidative damage by BER. Whether XPG also acts to enhance the repair of other DNA lesions that feed into the BER or ASR pathway is unknown at this time.

2.5.2. TCR of Oxidative Damage removed by the BER Pathway

Tg lesions have been shown to block ongoing transcription *(83,113,123)* and in normal cells are removed more rapidly from the transcribed strand of DNA than from the nontranscribed strand *(124).* The removal of Tg from DNA, facilitated by the enzymes of the BER pathway, is thus thought to be a transcription-coupled process.

Mutations which severely truncate the XPG protein, such as those seen in CS patients from XP group G, impair not only global, but also transcription-coupled removal of Tg lesions from DNA *(41).* In addition to activation of hNth1 glycosylase, XPG might interact with proteins required for TCR of oxidative lesions such as Tg. Other genes that encode proteins thought to be involved in TCR of Tg include CSA and CSB *(125);* BRCA1, the breast and ovarian cancer susceptibility gene *(77);* and hMSH2, a central component of the mismatch repair process.

Mismatch repair (MMR) is a DNA repair process that specifically ensures genetic stability by correcting DNA biosynthetic errors and by preventing recombination between divergent DNA sequences *(111,163,202).* In human cells, repair is initiated by one of two heterodimers: MutSα and MutSβ. The former, comprising hMSH2 and hMSH6, recognizes single mispairs, single-base loops and loops of two base pairs, whereas the latter, comprising hMSH2 and hMSH3, recognizes primarily three and four base-pair loops. When bound to repair targets in DNA, MutSα or MutSβ recruit a third heterodimer, MutLα, containing the proteins hMLH1 and hPMS2, to initiate repair *(136).* Cells defective in the hMSH2 gene lack MMR activity and are deficient in the removal of Tg from the transcribed strand of an active gene without an apparent decrease in overall genomic repair or an increased sensitivity to ionizing radiation *(124).* Conversely, cells defective in the hMLH1 gene show normal levels of TCR. Thus hMSH2 but not hMLH1 plays a role in the TCR of oxidative damage in DNA facilitated by the enzymes of ASR.

2.5.3. AP Endo and Poly(ADP-ribose)Polymerase in BER and DSBR

As discussed earlier, AP endo is a central component of the BER pathway. Recent evidence suggests that it may also play a role in the repair of certain double-strand breaks in DNA. Oxidative damage to the sugar moiety of DNA results in strand breaks containing a 3′-blocking group. The 3′-phosphodiesterase activity of human AP endo may function in the removal of such blocked termini at double-strand break ends. Additional factors may be required for the repair of some damaged 3′-termini, in particular those on 3′ overhangs *(234).*

PARP is an abundant nuclear protein that binds to single-strand interruptions in DNA that result from treatment with ionizing radiation or alkylating agents *(48).* PARP functions in DSBR when it stimulates DNA-PK, which is required for the rejoining of double-stranded DNA breaks *(213).* In terms of ASR, PARP contains a distinct binding site for the XRCC1 protein: a component of BER that binds both pol β and ligase III. By interacting directly with XRCC1, PARP may be involved in recruiting BER components such as pol β and ligase III to the site of DNA strand breaks *(149).* (Note that AP endo is not itself a substrate for DNA-PK; *see* Subheading 2.2.1.) The interaction between PARP and AP endo that function as components of both BER and DSBR further extends the ever-emerging list of shared molecular partners in DNA repair.

2.5.4. PCNA is Involved in all Three Major DNA Repair Pathways

Overlapping function for components of BER and other DNA-repair systems extend beyond removal of oxidative DNA damage to components involved in replication such as PCNA and pol δ/ε *(99).* In addition to its function in replication, PCNA has been found to play a part in several of the major cellular DNA repair systems including long-patch BER, NER, and MMR.

FEN1 is a structure-specific nuclease that recognizes and cleaves 5′ overhang or flap DNA structures *(81,143,144,207).* As reviewed earlier, FEN1 nuclease removes the 5′ reaction intermediate generated during long-patch BER. PCNA binds directly to FEN 1 and stimulates its endonucleolytic activity at branched structures and its exonucleolytic activity at nicked and gapped structures *(37,128,269).* Human PCNA was found to stimulate long-patch BER either through its direct interaction with the FEN1 nuclease or through its association with high molecular-weight polymerases such as δ or ε, which incorporate several nucleotides during strand displacement as part of the long-patch BER process *(128).*

PCNA also plays a role in other repair pathways. In NER, for example, DNA synthesis that follows excision of damaged DNA is mediated by pol δ or ε holoenzyme and requires PCNA *(178,224).* Furthermore, purified human PCNA interacts directly with expressed human XPG endonuclease, which interestingly shares homologous regions, including the predicted nuclease domain and a PCNA-binding region, with the FEN1 nuclease *(72).* A conserved arginine in XPG (Arg^{992}) is critical for PCNA binding and R992A and R992E mutant forms of XPG fail to reconstitute fully NER activity in vivo. The specific function of a PCNA-XPG interaction is not clear. The complex might provide a mechanism by which excision and resynthesis of NER could be interconnected. Could a PCNA-XPG complex also play a role in the BER of thymine glycol?

Recently it was demonstrated that pol δ is required for human MMR in vitro and that the resynthesis step of the pathway is PCNA-dependent *(138).* When MMR was examined in human cell extracts using an assay that did not require DNA synthesis, repair activity was inhibited by addition of $p21^{Cip1/Waf1}$ or a p21 peptide known to sequester PCNA *(247).* These data suggest DNA repair that PCNA plays a role in an early step of MMR, one that precedes synthesis.

More recent work has demonstrated that human MSH2, MLH1, PMS2, and PCNA can be co-immunoprecipitated, suggesting formation of a repair-initiation complex among these proteins *(80).* How PCNA functions in the early steps of MMR is not known but it has been suggested that it may help determine which strand of DNA should be repaired *(247).*

PCNA is a central player in all three of the major human repair systems, either as part of the resynthesis machinery, post-excision of damaged or mispaired bases in DNA, or as an integral part or the repair processes themselves. Indeed, sharing of common molecular partners such as PCNA, FEN1, and pol δ/ε between repair and replication-associated chain elongation might form the basis of a mechanism to allow coordination of DNA replication and repair processes in vivo *(255).* Is it possible that competition for PCNA between replication and repair systems following DNA damage could facilitate the stalling of replication and allow for repair of damage?

2.6. ASR Occurs in the Mitochondrion as well as in the Nucleus

Eukaryotic cells synthesize the bulk of their ATP in mitochondria through oxidative phosphorylation. The presence of large amounts of $FADH_2$ and NADH as well as reactive oxygen species including hydrogen peroxide *(214)* arising during the conversion of O_2 to H_2O are a clear liability to the integrity of biologically important molecules in the mitochondrion. Consequently, eukaryotic mitochondria are rich in the enzymes

involved in ASR *(192).* An in vitro system purified from *Xenopus laevis* ovary mitochondria repaired a single abasic site via mitochondrial AP endo, DNA polymerase γ, the DNA polymerase located in the mitochondrion, and a mitochondrial ligase, which is structurally related to DNA ligase III. Furthermore, a mitochondrial UDG was able to initiate repair of an oligonucleotide containing a single uracil. The dRPase was located on pol γ *(138).* Interestingly, when oxidative damage was induced by photoactivated methylene blue and the rate of repair examined in regions of the mitochondrial genome undergoing transcription, there was no difference in the rate of repair between strands or between two different regions of the genome that differ with regard to transcriptional activity *(3).* Consequently, it would appear that mitochondria are rich in enzymes involved in ASR and that repair is not transcription-coupled.

2.7. Unanswered Questions

In the quest to accurately depict the extent of knowledge about abasic site repair, this review leaves many unanswered questions. These include:

1. Is long-patch repair with polymerases other than pol β significant in normal cell physiology?
2. Given that the only physical biochemical evidence for interaction between different pairs of molecular partners is between ligase I and pol β, how do the molecules involved in ASR communicate with each other? How do they recruit one another?
3. Which protein or proteins is (are) responsible in the cell for dRPase activity when the phosphodeoxyribose remains associated on the upstream side of the lesion? AP endo can perform this function, but the turnover number is low and the activity is inefficient.
4. Which protein or proteins is (are) responsible in the cell for dRPase activity when the dRP remains associated on the downstream side of the lesion? Although pol γ performs this function in mitochondria, is it always pol β in nuclear ASR?
5. What regulates which pathway is chosen for repair? Is it the form of the substrate molecule independent of the lesion, e.g., short linear vs long linear vs circular DNA molecules? Is it the lesion itself or the glycosylase that is attracted to the lesion? If the glycosylase determines the pathway for repair, then there must be some way for the glycosylase to communicate with the appropriate DNA polymerase or with a co-factor that will bind to that DNA polymerase. In the case of long-patch repair, is the requirement for RFC as opposed to RPA a function of substrate (covalently closed circles with a single abasic site *[105,232]* vs a linear oligomer nucleotide with a single abasic site *[47]*)?
6. Once repair is in progress, can the pathway be switched? Although it is unlikely that long-patch repair might switch to short-patch repair, the possibility that the phosphodeoxyribose might be damaged after incision with AP endo would require the switch from short-patch to long-patch repair.
7. Are there proteins in ASR that remain to be discovered?
8. What regulates the distribution of different enzymes and pathways to different organelles?
9. Are there genotoxic insults from the environment in addition to oxidizing agents that might stimulate or decrease ASR?
10. We have no understanding at all of how these repair pathways will function on DNA packaged into chromatin or whether the type of chromatin (euchromatin or heterochromatin) will matter.
11. Are there cell-cycle effects that have gone undetected?

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4

Structure and Functions of the Major Human AP Endonuclease HAP1/Ref-1

Ian D. Hickson, Michael A. Gorman, and Paul S. Freemont

1. INTRODUCTION

Apurinic/Apyrimidinic (AP) sites arise spontaneously in DNA even at neutral pH owing to the inherent lability of the N-glycosyl bond. This lability is a consequence of the absence of the sugar 2′ oxygen in DNA compared to RNA. It has been calculated that up to 10,000 bases (primarily purines) are lost per human cell per day (reviewed in *3,18,39,40,68*). Because unrepaired AP sites are potentially both cytotoxic and mutagenic (noncoding), this burden of damage to DNA represents one of the major threats to viability and genome stability in human cells. AP sites in DNA can also arise either by the actions of reactive oxygen species (ROS), or by enzymatic excision of damaged bases via the cleavage of the N-glycosyl bond catalyzed by a DNA glycosylase. AP sites in double-stranded DNA are recognized by a class of enzymes termed AP endonucleases that cleave the phosphodiester backbone on the 5′ side of the AP site via a hydrolytic mechanism and hence catalyze the initial step in AP site repair (reviewed in *3,18,68*). A number of DNA glycosylases exhibit AP site-cleavage activity as part of their mechanism of action. However these enzymes act as β-elimination catalysts, cleaving the phosphodiester backbone 3′ to the AP site. This class of enzyme (so-called class I AP endonucleases) will not be discussed further in this chapter. Instead we direct readers to recent reviews containing a discussion of the properties of these enzymes *(13,14,22,37,45,59,69).*

In all organisms that have been analyzed, the hydrolytic AP endonucleases (the class II enzymes) perform roles in addition to AP site repair. In particular, these enzymes act as phosphodiesterases to remove atypical moieties (i.e., other than 3′ hydroxyl) from the 3′ termini of DNA strand breaks induced by ionizing radiation and other DNA damaging agents that generate ROS *(3,16,18,59,68).* These atypical termini, such as 3′ phosphoglycolate and 3′ phosphate, are generated through attack on the sugar moiety of DNA, generating a break in the phosphodiester backbone but leaving a fragment of the sugar at the 3′ terminus of the break. Because DNA polymerases require a 3′-OH terminus to prime DNA repair synthesis, removal of these 3′ blocking lesions is essential to allow repair of oxidative DNA damage to be completed. This chapter focuses on the major AP endonuclease/phosphodiesterase in human cells, called HAP1 (also called APE1, Ref-1, and APEX) (*see 3,6,16,55* for previous reviews). In particular, we shall

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review our current knowledge about the structural basis of AP site recognition and repair by HAP1. A wider review of the base excision repair (BER) pathway in which HAP1 participates is discussed in Chapter 3 by Strauss.

In addition to its ability to recognize and participate in the repair of several classes of DNA lesions, HAP1 also possesses an apparently unrelated activity that can regulate the reduction/oxidation (redox) state of cellular proteins and so modulate their ability to bind to DNA. We shall discuss how this "redox" function might be relevant in the cellular response to oxidative stress.

2. THE HAP1 FAMILY OF AP ENDONUCLEASES

HAP1 belongs to family of structurally and functionally conserved enzymes that has members in bacteria, unicellular eukaryotes, insects, plants, and mammals (Fig. 1). The best-characterized member of the family is *Escherichia coli* exonuclease III (the *xth* gene product; ref *58*), a multifunctional protein that is used extensively in laboratories as a molecular biology reagent. Exonuclease III can be considered to represent the "core" DNA repair-domain characteristic of the family and the only region of the larger eukaryotic family members that is highly conserved. Indeed, this repair domain is one of the most highly conserved protein "modules" yet identified, with at least 50% sequence similarity (when conservative changes are included) evident throughout the family. In addition to HAP1, mammalian-cell homologs of exonuclease III have been identified from bovine and rodent sources *(17,52,53,60,74).* These homologs show a very high level of sequence identity with HAP1 (>90%); the majority of the differences being found within the N-terminal 62 amino acid region.

The largest family member is Rrp-1 from *Drosophila (41,57),* which contains a 427 amino acid N-terminal portion that is not conserved in any other known AP endonuclease (Fig. 1). This domain has been implicated in binding single-stranded DNA and in DNA renaturation. The extensive N-terminal domain of the *Arabidopsis* Arp protein *(2)* is structurally unrelated to that in Rrp-1 and has not been implicated to date in any functions related to DNA metabolism. In contrast, Apn2 and SPBC3D6.10, the recently identified budding and fission yeast homologs of HAP1 *(34),* include a C-terminal domain that is not a feature of exonuclease III or any other known eukaryotic family member (Fig. 1). The role(s) of these C-terminal domains awaits further analysis. The yeast homologs show a lower level of sequence similarity to the other family members, but the key catalytic residues (*see* Subheading 5.2.) are conserved in these enzymes.

HAP1 contains two other functionally defined domains, located N-terminal to the highly conserved DNA repair domain (Fig. 1). The "redox" activity of HAP1, through which it modulates transcription factor function (*see* Subheading 5.), requires residues within the region between amino acids 36 and 82. This domain overlaps the DNA repair domain, which commences at around residue 62. At position 65 within this redox domain is a cysteine residue that has been shown to be essential for redox activity *(67).* A cysteine residue located in an approximately equivalent position is found in the Rrp-1 and Arp proteins, but is absent from the other family members.

The short, extreme N-terminal portion of HAP1 (residues 1–36) is found only in the family members isolated from mammalian species. This domain is essential for the targeting of HAP1 to the nucleus (*see* Subheading 7.).

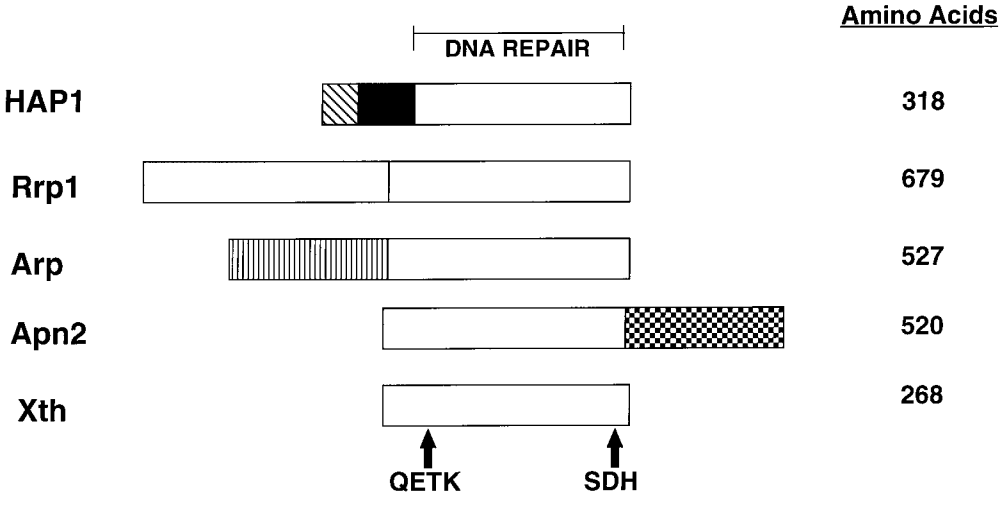


Fig. 1. Schematic representation of the structure of selected members of the HAP1 family of AP endonucleases. The proteins shown are HAP1 *(H. sapiens),* Rrp-1 *(D.melanogaster),* Arp *(A. thaliana),* Apn2 (*S. cerevisiae)* and Xth *(E. coli).* The proteins are aligned by their DNA repair domain, which is shown as an open box and indicated above. The defined "redox" domain of HAP1 is shown as a black box, and the nuclear targeting domain of HAP1 is shown by the diagonally hatched box. The N-terminal domains of Rrp-1 and Arp, which are absent from the other members and are not homologous to each other, are shown with gray shading and vertical hatching, respectively. The C-terminal domain of Apn2, which is absent from the other members is shown as a checked box. The number of amino acid residues in each protein is shown on the right. The positions of the QETK and SDH motifs, which contain the invariant glutamate and histidine residues (Glu-96 and His-309 in HAP1) that are crucial for metal ion binding and catalysis, respectively, are indicated below the Xth protein. See text for details.

3. DNA REPAIR FUNCTIONS OF HAP1

3.1. Substrates for HAP1

Exonuclease III has long been known to be a multifunctional enzyme that possesses AP endonuclease, 3′ phosphodiesterase, 3′ phosphatase, RNaseH, and 3′–5′ exonuclease activities *(24,50,51,54,70).* However, a functional significance for some of these activities remains elusive. In particular, no role for the RNaseH or exonuclease activity has been identified. Exonuclease III is the major AP endonuclease in *E. coli,* and cell extracts from *xth* mutants have much-reduced levels of AP-site nicking activity *(24,70).* Consistent with this, *xth* mutants are sensitive to monofunctional alkylating agents that generate AP sites following glycosylase-mediated excision of alkylated bases *(44).* Evidence that the 3′ phosphodiesterase and phosphatase activities of exonuclease III are important in vivo comes from the finding that *xth* mutants are also sensitive to oxidant chemicals *(15),* particularly hydrogen peroxide, that generate DNA strand breaks terminating in a fragmented sugar group.

In contrast to exonuclease III, for which rates of phosphodiester bond cleavage for its various enzymatic activities are broadly similar, HAP1 can be considered to have evolved as a more specialized repair enzyme. HAP1 is a particularly powerful AP endonuclease, but is 100- to 1,000-fold less efficient (based on catalytic rates) than

exonuclease III as a phosphodiesterase, phosphatase, RNaseH, or exonuclease enzyme *(4,5,10,60,73).* This calls into question the biological significance of these additional activities in HAP1. Nevertheless, our original purification of BAP1, the bovine homolog of HAP1, was on the basis of monitoring 3′ diesterase activity against phosphoglycolate residues generated by bleomycin, and it was clear during those experiments that BAP1 represents the major 3′ phosphoglycolate diesterase activity in extracts from bovine calf thymus *(53).* Similarly, the mouse homolog of HAP1, termed APEX, was purified on the basis of this activity, and APEX has been demonstrated to be the most powerful 3′ diesterase activity in mouse nuclei *(60–62).* Although the 3′ phosphodiesterase activity of HAP1 is likely to have some functional significance, the enzyme is unable to remove 3′ phosphoglycolate residues from a 1 or 2 bp protruding 3′ terminus at a DNA strand break *(64),* suggesting that additional proteins are required for the repair of at least some forms of 3′ blocking lesions. HAP1 is competent, however, for removal of these lesions from blunt-ended or 3′ recessed ends. Consistent with an apparent requirement in human cells for additional 3′ phosphodiesterases, at least one activity distinct from HAP1 that can remove 3′ blocking lesions from oxidatively damaged DNA has been partially purified *(10,75).* However, this enzyme has eluded identification to date.

3.2. HAP1 is a Functional Homolog of Exonuclease III

Expression of the HAP1 cDNA in *xth* (exonuclease III-deficient) and *xth nfo* (exonuclease III and endonuclease IV deficient) strains of *E. coli* has been shown to confer at least partial resistance to DNA-damaging agents, indicative of functional complementation *(11,16,52).* Consistent with a key role as an AP endonuclease rather than as a 3′ phosphodiesterase, HAP1 expression can complement the sensitivity of these mutants to alkylating agents, but not to hydrogen peroxide. Nevertheless, some degree of acquired resistance to γ-rays has been observed in these experiments *(12),* suggesting that HAP1 can function as a phosphodiesterase in vivo.

3.3. Kinetics of AP Site Cleavage by HAP1

The kinetic parameters for cleavage at AP sites by HAP1 have been quantified in several different laboratories. For example Strauss et al. *(63)* studied cleavage at an AP site generated by excision of a uracil residue in an oligonucleotide substrate, and found values for K_m and K_{cat} of 100 nM and 600 min^{-1}, respectively. With our preparations of HAP1 and a similarly generated AP site-containing oligonucleotide substrate, we obtained values of 47 nM for K_m and 150 min^{-1} for K_{cat} *(83).* Erzberger et al. *(20)* have also detailed the kinetics of cleavage at AP site analogs, including the frequently studied tetrahydrofuran (THF) residue, and obtained values for this residue of 27 nM and 40 min^{-1}. In that study, other analogs, such as propanediol and a branched abasic structure, were less efficiently cleaved, with lower K_{cat} values. Thus, there is general agreement that the specificity constant of HAP1 (defined as K_{cat} divided by K_m) is in the range 3–6 min^{-1} nM^{-1} for "regular" AP sites, but is several-fold lower for AP site analogs. These in vitro experiments using oligonucleotide substrates have also shown that in order to recognize AP sites efficiently, HAP1 requires at least 4 bp 5′ and 3 bp 3′ to the lesion. As part of the recognition process, HAP1 makes contacts with both the major and minor grooves and with both the AP site containing strand and the opposing strand of the DNA *(72,73)* (*see* model in Subheading 5.).

3.4. Binding of HAP1 to AP Sites

Treatment of HAP1 with EDTA eliminates AP endonuclease activity, but does not prevent binding of the enzyme to its substrate. This observation has been exploited to study determinants of AP site recognition by HAP1 and the half-life of HAP1:DNA complexes upon addition of Mg^{2+}. Rothwell and Hickson *(56)* and Wilson et al. *(72)* demonstrated that addition of ethylene diamine tetraacetic acid (EDTA) extended the half-life of HAP1:AP site complexes to a degree that permitted detection of the complex using gel-retardation assays. Using this methodology, the highly conserved Asn-212 residue was shown to be important for AP site recognition by HAP1 *(20).* Wilson et al. *(72)* further showed that the phosphodiester backbone in the HAP1:DNA complex is intact, but can be cleaved rapidly upon addition of Mg^{2+}. Consistent with this, Strauss et al. *(63)* calculated the rate of product release to be extremely rapid and proposed that the reaction mechanism for HAP1 follows the scheme proposed by Briggs-Haldane, in which the forward catalytic reaction competes with the dissociation of the enzyme-DNA complex. Recent data have extended this model and suggest that Mg^{2+} plays roles both in catalysis and in substrate interactions *(42).*

3.5. HAP1 Repairs Oxidized AP Sites and Some Bulky DNA Adducts

HAP1 is efficient at repairing not only regular AP sites, but also oxidized AP sites generated by ionizing radiation and radiomimetic chemicals. DNA containing one form of oxidized AP site, the C-4-keto-C-1-aldehyde generated by bleomycin, is cleaved by HAP1 with an efficiency close to that for non oxidized AP sites *(80).*

Several agents, including ionizing radiation, can generate multiple DNA lesions in close proximity. Hence, cleavage at oxidized AP sites generated in this way has the potential to generate double-strand breaks in DNA, a much more cytotoxic lesion than any individual oxidative lesion. Chaudhry and Weinfeld *(9)* showed that the ability of HAP1 to cleave at AP sites closely spaced on opposite strands of a DNA duplex is influenced by the relative positions of the two lesions. AP sites positioned 3′ to each other can be cleaved, albeit less efficiently that single lesions, but those placed 5′ to each other are either not cleaved at all, or only one of the two strands is cleaved efficiently.

Although the BER pathway of which HAP1 is a constituent is generally considered to be specific for "small" lesions in DNA, unlike the nucleotide excision repair pathway, Hang et al. *(27)* showed that HAP1 is involved in the repair of a bulky carcinogenic adduct in DNA. Reaction of *p*-benzoquinone (a benzene metabolite) with DNA generates several lesions including 3,N^4 benzetheno-2′-deoxycytidine (*p*-BQ-dC). This lesion has no associated AP site. HAP1 cleaves DNA 5′ to this lesion, leaving the adduct bound to the 5′ residue of the cleaved DNA. Further work on mutant HAP1 proteins lacking residues important for AP endonuclease activity, indicated that the pBQ-dC endonuclease activity utilizes the same active site as that used for AP site cleavage *(26).*

4. REDOX FUNCTIONS OF HAP1

4.1. The Phenomenon of Redox Regulation of Proteins

Fos-Jun dimerization forms a bipartite DNA-binding domain that interacts with pseudopalindromic AP-1 binding sites, and with palindromic CRE sites *(31).* Regulation of Fos-Jun DNA binding activity has been associated with a "redox" process medi-

ated by the reduction of a conserved cysteine residue located in the DNA binding domain of Fos-Jun heterodimers and Jun-Jun homodimers *(1)*. DNA binding and transcriptional activation by such complexes are inhibited in an oxidizing environment, with functional activity of Fos-Jun heterodimers and Jun-Jun homodimers being restored by the addition of reducing agents such as dithiothreitol (DTT). Substitution of Cys-154 in Fos or Cys-272 in Jun with serine, results in an increase in DNA binding activity (and a concomitant loss of redox regulatory control), suggesting that Fos-Jun activity is mediated by the reduction of cysteine residues *(31)*. A similar redox regulatory system has been shown for p53 (*see* Subheading 4.3.).

4.2. The N-terminal Region of HAP1 Mediates Redox Regulation of Fos-Jun

The connection between the regulatory phenomenon described earlier and HAP1 came from work in the Curran laboratory showing that a factor present in human cells could substitute for DTT in stimulating DNA binding of oxidized Fos-Jun *(76)*. Isolation of cDNAs encoding this factor, termed Ref-1 by these authors, demonstrated that it was identical to HAP1 *(78)*. The region of the HAP1 protein that confers redox activation of Fos and Jun proteins has been mapped in two studies through an analysis of truncated versions of the HAP1 protein *(67,77)*. These studies gave similar results in that C-terminal truncation of HAP1 eliminates DNA repair, but not redox activity, whereas truncation from the N-terminus has the opposite effect. Fine mapping of the region responsible for redox function indicated that the first 36 residues of HAP1 are dispensible, but that the "domain" between amino acids 36 and 82 is essential *(67,77)*. Indeed, our unpublished data indicate that a polypeptide comprising residues 1–82 possesses redox activity, suggesting that residues present in the core DNA repair domain beyond residue 82 are unlikely to be critical for the redox function of the HAP1 protein in vivo.

To date, only one amino acid residue has been implicated in the redox function of HAP1. A site-directed mutagenesis study focusing on the putative role of cysteine residues in HAP1 identified Cys-65 as important for regulation of Jun DNA binding *(67)*. Substitution of Cys-65 by alanine results in a significant loss of HAP1 redox activity. Whether this effect is a direct one, indicating that cysteine-65 might be the redox active site, is unclear at this stage, particularly considering the fact that the crystal structure of HAP1 indicates that cysteine-65 is not surface exposed (*see* Subheading 5.). These data suggest either that a conformational change in HAP1 is required in order for it to direct interactions with transcription factors, or that the negative effect on redox activity of the Cys-65 to alanine substitution is mediated through altering the tertiary structure of HAP1. Further analyses will be required to distinguish between these possibilities.

4.3. p53 is Regulated by HAP1

A great deal of interest has been generated by the finding that p53 is one of the apparent targets for regulation by HAP1. Jayaraman et al. *(33)* purified a protein that could convert a "latent" form of p53 that is inert for DNA binding into a form that could activate transcription in vitro. Analysis of this protein indicated that it is identical to HAP1. Interestingly, these authors suggested that HAP1 plays two roles in the regulation of p53 function, one redox activity-dependent and one independent of this activity. They found that HAP1 can stimulate the DNA binding activity of oxidized forms of

both full-length p53 and a C-terminally truncated version (p53Δ30). However, in the presence of DDT, HAP1 potently stimulates binding by full-length p53, but not by truncated p53. Interestingly, unlike the activation of Fos-Jun dimers, which seems to require stoichiometric amounts of HAP1, stimulation of p53 by HAP1 is surprisingly potent, with half maximal stimulation being observed at a molar ratio of HAP1 to p53 of approx 1:200 *(33).* The functional relevance of this interaction has also been demonstrated by the observation that HAP1 can enhance the ability of p53 to transactivate gene expression *(33).*

4.4. Relevance of Redox Control In Vivo

To date, there have been only a limited number of studies that have addressed the question of whether this form of redox regulation of transcription factors actually exists in vivo and, moreover, whether it is relevant to the regulation of gene expression. One study suggesting that redox regulation is biologically important utilized a mutated Fos protein in which the critical cysteine residue in the DNA binding domain was replaced by a serine *(47).* Retroviral-mediated expression of the serine-substituted Fos protein in chicken-embryo fibroblasts caused a three-fold increase in AP-1 DNA binding activity compared to controls expressing the nonmutated Fos protein. Moreover, viruses expressing the mutant Fos protein gave increased numbers of transformed colonies and a general increase in colony size compared to controls, suggesting strongly that the ability to escape redox control enhances the transforming capabilities of the Fos protein *(47).*

Thioredoxin is a multifunctional protein that can mediate redox control of numerous cellular processes via a cysteine-dependent redox active center. It has been shown that the ability of HAP1 to modulate Fos-Jun DNA binding is enhanced by thioredoxin *(30),* suggesting that thioredoxin may activate HAP1 through its ability to maintain HAP1 in a reduced and consequently active state. Using in vitro cross-linking and a mammalian cell two-hybrid system, Hirota et al. *(30)* demonstrated that HAP1 and thioredoxin are able to associate in a complex and that this association requires the redox active center of thioredoxin. These data suggest that HAP1 and thioredoxin form part of a redox-mediated cascade that is important for regulation of gene expression in mammalian cells.

5. STRUCTURE OF HAP1 AND CHARACTERIZATION OF THE DNA REPAIR ACTIVE SITE

5.1. Structure of HAP1

The crystal structures of HAP1 *(23)* and exonuclease III *(46)* have been determined at 2.2Å and 1.7Å resolution, respectively. Not suprisingly, given their primary sequence similarity, HAP1 and exonuclease III show strong conservation of tertiary structure. In order to generate usable crystals of HAP1 *(23),* it was necessary to delete the N-terminal 35 amino acids. Hence, the discussion of the HAP1 structure given below relates to a molecule comprising residues 36–318 (designated $HAP1^{36–318}$). This truncated version of HAP1 is, however, fully functional, with no apparent reduction in AP endonuclease or redox activity *(67).* $HAP1^{36–318}$ in the presence of the calcium analog, samarium, produced high-quality crystals *(23).*

With the exception of the region between residues 36 and 43, $HAP1^{36–318}$ is a globular α/β protein consisting of two domains (domain 1. residues 44–136 and 295–318.

domain 2. 137–260 and 282–294), with overall dimensions 40 Å × 45 Å × 40 Å. Both domains display similar topologies, each comprising a six-stranded β-sheet surrounded by α-helices, which pack together to form a four-layered α/β-sandwich. Each β-sheet is composed of a pair of three-stranded anti-parallel β-motifs, with the β-strands in both domains being flanked by topologically equivalent α-helices. Strands β_1/β_7 are flanked by kinked α-helices, α_1–α_2/α_5–α_6, in domains 1 and 2, while β_3/β_8 are flanked by short α-helices, α_3/α_4, respectively. Helices α_7, – α_{10} have no comparable structure in domain 1, while α_{12} forms an inter-domain helix. Residues 261–281 form two extra-domain anti-parallel β-strands, β_{10} and β_{11}, separated by a helical turn, α_{11}.

An important insight into the catalytic mode of action of HAP1 came from the finding that its structure is also very similar to that of bovine DNase I *(23).* This is despite the fact that DNase I and HAP1 show <20% primary sequence homology. HAP1, exonuclease III and DNase I not only illustrate an overall conservation of the protein fold (Fig. 2), but also a conservation of residues shown by site-directed mutagenesis to be important for catalysis (*see* Subheading 5.2.). It is clear from these comparisons that the mechanism by which these enzymes cleave the phosphodiester backbone of DNA is probably very similar. We have proposed a model *(23)* for how HAP1 binds to DNA based on the structure of DNase I bound to a DNA octamer duplex *(38).* In this model, HAP1 is proposed to make only a limited number of specific protein/base interactions, and instead to bind DNA mainly via phosphate and nonspecific hydrophobic interactions.

5.2. Active Site of HAP1 and Proposed Catalytic Mechanism

The active site of HAP1 lies in a pocket at the base of the α/β-sandwich and is surrounded by loop regions. Within the active site, the imidazole ring of His-309 interacts with the carboxylate of Asp-283, which in turn forms a hydrogen bond with Thr-265. The side chains of Tyr-171 and Glu-96 are hydrogen bonded, as are those of Asn-68 and Asp-210, with the side chain of Asp-210 also being hydrogen-bonded to the main-chain amide of Asn-212. Together with the hydrogen bonding of Asp-70 to Lys-98, these interactions form an intimate hydrogen-bonding network within the active site. A single metal ion binds to the side-chain of Glu-96 (mean distance of 2.5Å from the carboxyl group).

During catalysis, it is proposed that His-309 of HAP1 acts as the general base to abstract a proton from a water molecule, whereas Asp-283 orientates the imidazole ring of His-309 and stabilizes its transiently positive-charged state (Fig. 3). The resulting hydroxide ion then attacks the scissile AP 5′-phosphate via an inversion of configuration. The catalytic importance of His-309 and Asp-283 have been demonstrated, because mutation of His-309 to Asn and Asp-283 to Ala results in a dramatic reduction in catalytic activity *(4).* However, recent mutagenesis work has suggested that Asp-283 is not essential for AP endonuclease activity and that its role may be substituted by Asp-308, because the double mutant is markedly more defective in AP endonuclease activity than is either single mutant *(43).* This is possible, because the carboxyl group of Asp-308 is only 3.2Å from His-309. The transition state intermediate could be stabilized by the divalent metal ion bound to Glu-96, in a similar way to that proposed for DNase 1 *(71).*

Recently, Mol *et al. (82)* reported the cocrystal structure of HAP1 bound to oligonucleotides containing a single, synthetic abasic site (tetrahydrofuran). Their data provide new insights into both the catalytic mechanism of action and the mode of AP site recognition by HAP1 (*also see* discussion below). Mol *et al.* propose that the hydroxyl nucle-

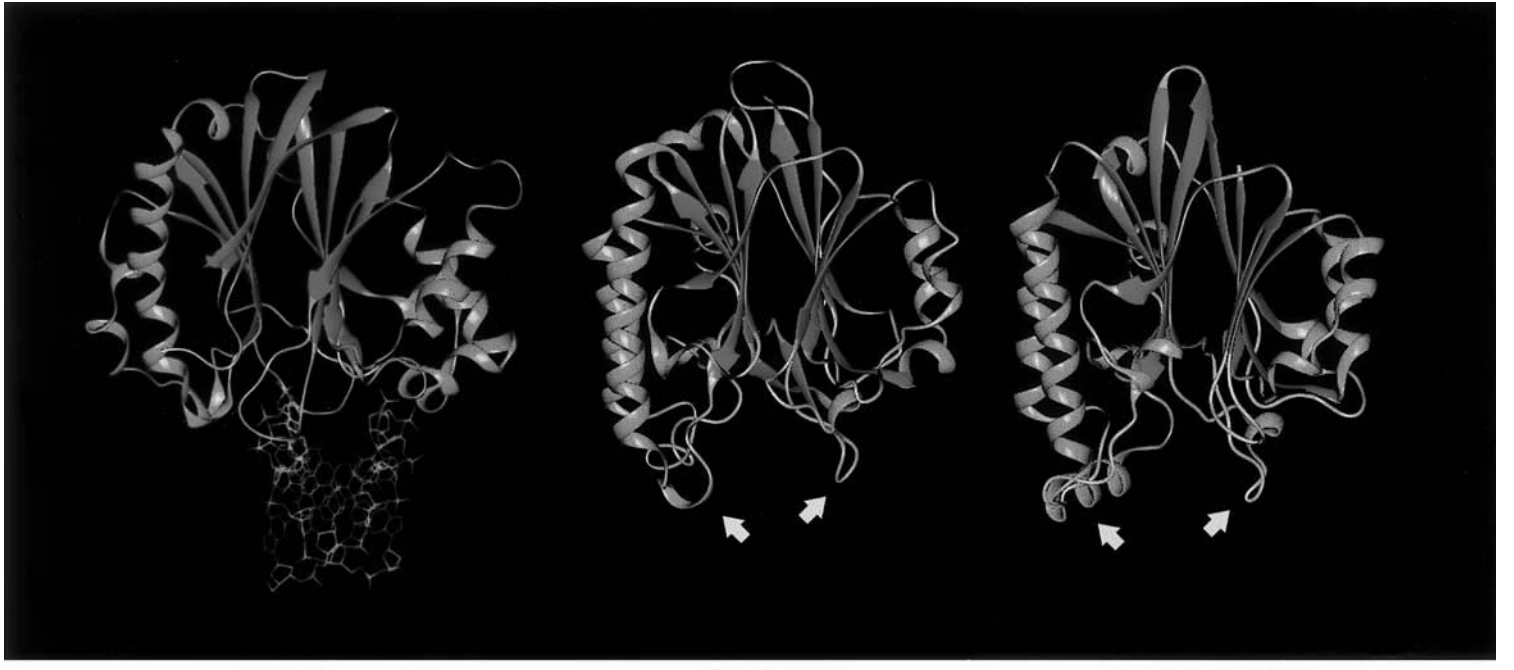


Fig. 2. Structural similarity between the overall folds of DNase I, HAP1, and exonuclease III. Ribbon representation of DNase I, HAP1, and exonuclease III, with α-helices colored blue and β-strands colored pink. DNase I is shown complexed to DNA, (atom colored stick model). The yellow arrows indicate the three helical loop regions that are present in HAP1 and exonuclease III, but are absent from DNase I (*see* text). Figure produced using PREPI (courtesy of S. Islam and M Sternberg, ICRF).

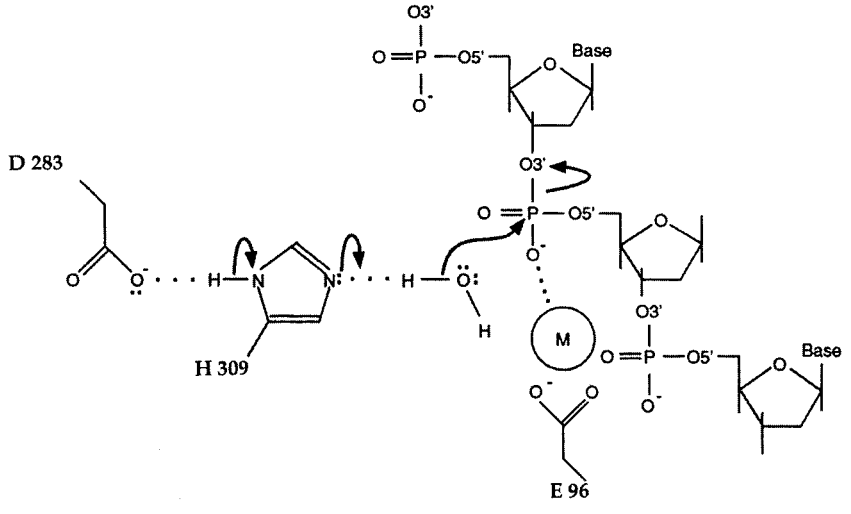


Fig. 3. Proposed catalytic mechanism for HAP1. His-309 acts as general base and abstracts a proton from a water molecule. The hydroxide ion thus formed then attacks the phosphate group 5′ to the AP site. A divalent metal ion (M), bound to Glu-96, could be involved in stabilizing the transition state intermediate.

ophile is generated by the Asp-210 residue, which is oriented by hydrogen bonding with Asn-212, and Asn-68, and that His-309, Asn-212 and Asn-174 act to correctly orient the target AP site 5′ phosphate. In this scheme, the role of the metal iron is to stabilize the transition state intermediate and the O3′ leaving group. Consistent with this proposed scheme, mutation of Asp-210 to Ala or Asn eliminates AP endonuclease activity *(83,84).* Nevertheless, these mutated proteins are still competent for AP site binding *(83,84).*

5.3. AP Site Recognition by HAP1

The solution structures of several oligonucleotides containing various AP site analogs have been determined by nuclear magnetic resonance (NMR). By superimposing the scissile phosphodiester bond located at the active site of HAP1 from the model by Gorman et al. *(23)* on the averaged NMR structure of DNA containing an αAD (apyrimidinic duplex) moeity *(7),* potential interactions between HAP1 and the DNA duplex can be proposed. In this model (Fig. 4), the helical loops α5 (residues 176–181) and α11 (residues 267–277) are positioned within the major and minor grooves, respectively, making potential DNA-phosphate backbone interactions. A third region, α8 (residues 222–227; 164–173 in exonuclease III) is also positioned within the major groove. Hydrophobic residues conserved in the AP endonuclease family (Leu-220, Phe-232, and Trp-280 in HAP1), seem to be important for stabilizing and/or positioning these loops. Interestingly, these three loops are not conserved in DNase I and have been proposed *(23)* to be a major determinant of the AP site specificity of HAP1 and exonuclease III, given that DNase I is a nonspecific endonuclease.

The primary sequence of HAP1 also shows similarity to L1 endonuclease (*see* discussion in ref. *23*), which forms part of human L1 elements, a highly abundant poly(A) (i.e., non-LTR) retrotransposon that contains highly repetitive DNA. L1 endonuclease,



Fig 4. Model of HAP1 bound to DNA. Ribbon representation of HAP1 with α helices shown in blue and β sheets in pink. Apyrimidinic duplex DNA (shown in the a hemiacetal conformation and taken from Berger and Bolton *[7]*) is represented as a gold surface generated using a probe size of 1.4 Å. Loop regions are predicted to lie in both the major and minor grooves of the DNA. The loop region α8, homologous to the one observed by Cal *et al. (8)* to impart AP endonuclease-like activity on Dnase I, is colored yellow. Figure produced using PREPI (courtesy of S. Islam and M Sternberg, ICRF).

like DNase I, has no specificity for AP sites, and 2 of the 3 helical loops specific for the AP endonucleases (α8 and α11) are absent from L1 endonuclease. To address the functional relevance of the α8 loop, Cal et al. *(8)* inserted the equivalent loop from exonuclease III (designated α_M) into DNase I. The modified DNase I (DNase-exohelix) acquired the ability to bind and cleave DNA at AP sites *(8),* albeit with a much lower efficiency than that seen with "native" AP endonucleases.

In HAP1, Phe-266 lies close to the active site and has been suggested to be important in the direct interaction with the ribose ring at AP sites *(23).* However, a Phe-266 –Ala mutant shows only a six-fold reduction in AP site-binding activity, suggesting that it is important, but not essential for AP site recognition *(20).* Given that AP sites containing nonring structures are recognized and cleaved by HAP1 *(73),* together with the results of Erzberger et al. *(20),* it seems likely that a number of other structural elements (in addition to the Phe-266 residue) found in the AP endonucleases, but not in DNase I, will be required for efficient recognition and repair of AP sites.

The cocrystal structure of HAP1 with tetrahydrofuran residues *(82)* has provided a novel and direct insight into the mode of AP site recognition by this enzyme. HAP1 inserts loops into both the major and minor grooves of DNA and, as predicted previously *(23),* binds flipped-out abasic deoxyribose in the active site. This stabilization of the flipped moiety is achieved by kinking of the DNA helix. Interestingly, the active site pocket created excludes one of the two racemic forms of the AP site, the β-anomer. The enzyme is locked onto the damaged DNA by insertion of Arg-177 into the major groove and Met-270 into the minor groove. Apparently at odds with these observations is the finding that substitution of Arg-177 or Met-270 by Ala does not eliminate endonuclease activity. Indeed, the R177A mutant has a 3-fold increased K_{cat}. Mol *et al*. *(82)* argue that this apparent anomaly reflects the greater importance attached to coordinating AP site cleavage with the subsequent steps of base excision repair, than to maximizing the rate of enzyme turnover. It may be important for HAP1 to remain bound to the product of its hydrolysis reaction, and not to dissociate from the nicked (and probably toxic) DNA intermediate.

5.4. Structure of the Redox Domain of HAP1

The "redox" domain of HAP1 is associated with the N-terminal region, including amino acids 43–62 *(67,77).* The structural analysis of HAP1 *(23)* shows that this region forms an extended loop which lies across the β-strands, β13 – β14. Cys-65 is also implicated in the "redox" activity of HAP1 *(67).* In the HAP1 crystal structure, Cys-65 is located on β1, with its side-chain pointing into a hydrophobic pocket (containing Trp-67, Trp-75, Trp-83, Pro-89, Leu-92, Leu-94, and Pro-311) and away from the central β-sheet. Solvent accessibility calculations with a probe size of 1.4 Å showed that Cys-65 is inaccessible to solvent and would, therefore, be unlikely to interact directly with residues from other proteins. Interestingly, Cys-93 and Cys-208 lie within the core β-sheet, with their side-chains only 3.5 Å apart, and are in close proximity to Cys-65. However, in the crystal structure, there is no evidence for the existence of a disulfide bond between them. However, incubation of diamide with HAP1 has been associated with the formation of an intramolecular disulfide bond *(77),* suggesting that Cys-93 and Cys-208 could form a disulfide bond under different crystallization conditions.

Removal of 50 or more N-terminal residues from HAP1 abolishes redox activity *(67,77).* Such truncations could be deleterious to the overall HAP1 fold and may account, at least partially, for the lack of redox activity. Several residues, particularly in the region comprising residues 47–59, make hydrogen bonding or salt-bridge interactions with the globular core of the enzyme *(23).* The role of Cys-65 in mediating the "redox" activity is perplexing, because it is buried in a hydrophobic pocket. However Cys-65 substitutions could disrupt this large hydrophobic-core region, adversely affect the stability and/or folding of HAP1, which then indirectly influences the apparent "redox" activity by altering the conformation of the N-terminal domain. It is also possible that the HAP1 crystal structure reported by Gorman et al. *(23)* is not representative of a "redox" active conformation. HAP1 must be in a reduced state to exhibit redox activity, and no attempt was made to maintain such a state during crystal growth. A reducing environment may promote a conformational change, which could then allow Cys-65 to become solvent-exposed.

6. ROLE OF HAP1 IN CELLULAR PROTECTION AGAINST CYTOTOXIC STRESSES

Targeted disruption of the gene encoding the murine HAP1 homolog results in early embryonic lethality *(79)* and therefore it has not been possible to derive any information from a mouse "knockout" model for HAP1 deficiency. As an alternative, several groups have studied cell lines in which HAP1 has been depleted following expression of *HAP1* antisense RNA. These studies have shown that HAP1 is required for cellular protection against a wide range of DNA damaging agents and oxidative stresses *(12,48,66)*. HAP1-depleted cells are sensitive to agents that generate AP sites via DNA methylation (such as methyl methane sulphonate; MMS), and to agents that generate oxidative DNA damage, such as x-rays, hydrogen peroxide, paraquat, bleomycin, and hyperoxia (95% O_2). These data are consistent with the known roles of HAP1 as an AP endonuclease and a 3′ phosphodiesterase (*see* Subheading 3.). Perhaps more surprisingly, HAP1-depleted cells are hypersensitive to hypoxic stress (1% O_2). However, the precise mechanism by which hypoxia kills cultured mammalian cells has yet to be delineated, and therefore it is not clear whether the mode of killing HAP1-depleted cells by hypoxia is in any way unique.

Unfortunately, all of the previous studies in this area have failed to discriminate between a DNA repair role and redox role for HAP1 in protecting cells against cytotoxic stresses. Indeed, the vast majority of the agents to which HAP1-depleted cells are sensitive are oxidizing agents that have the potential both to generate oxidative DNA damage and to modulate the redox status of cellular proteins. The finding that HAP1-depleted cells are hypersensitive to MMS, which, as far as we are aware, does not generate any oxidative stress, suggests that DNA repair deficiency underlies at least this phenotypic effect of disrupting HAP1 function. Nevertheless, it would not be surprising if a combination of deficiency in DNA repair and redox functions were important for the pleiotropic radiation and drug sensitivity of cells expressing HAP1 antisense RNA. Evidence to substantiate this suggestion will only be forthcoming when targeted mutations in *HAP1* or one of its mammalian homologs are generated that inactivate separately either the redox function or the repair function.

7. SUBCELLULAR LOCALIZATION OF HAP1

7.1. Immunohistochemical Staining for HAP1

Although the putative redox function for HAP1 could theoretically be performed in any cellular compartment, it is clear that HAP1 must accumulate in the nucleus to effect its role as a DNA repair enzyme. Despite this, a number of immunohistochemical studies have shown that HAP1 is not necessarily exclusively nuclear, but instead is sometimes distributed in both the nucleus and cytoplasm, or indeed can be exclusively cytoplasmic in some cell types. For example, Kakolyris et al. *(36)* studied HAP1 expression level and subcellular localization in normal colorectal mucosa, as well as in hyperplastic polyps, adenomas, and carcinomas of the colon. This study indicated that HAP1 is located in different cellular compartments depending on the extent of differentiation of the cells. In the poorly differentiated cells located in the lower portion of colonic crypts, staining for HAP1 is predominantly nuclear, whereas it is cytoplasmic in the more differentiated superficial colonic epithelium. Moreover, HAP1 is partially

or exclusively localized to the cytoplasm in 90% of adenomas and carcinomas of the colon, and in stromal macrophages. HAP1 staining is exclusively nuclear in stromal fibroblasts and endothelial cells. A similar pattern of marked changes in subcellular localization depending on differentiation state of the cells has been reported by Duguid et al. *(19)* in a study of duodenal tissue. These authors showed that HAP1 is localized to the nuclei of cells in the duodenal crypts and proximal villi, but not in the distal villi. Thus, differential localization of HAP1 is seen in cells from both the proximal and distal intestine.

In a separate study, Kakolyris et al. *(35)* showed that HAP1 has a uniformly nuclear localization in normal breast epithelium, but it is predominately cytoplasmic in lactating and glandular epithelium. In breast carcinomas, HAP1 expression in the nucleus seems to be related to advanced differentiation, which is well-established to relate to good prognostic features. Consistent with those findings, evidence of exclusive localization of HAP1 to the nucleus significantly correlates with negative lymph-node status. In contrast, cases with cytoplasmic as well as nuclear staining showed an association with lymph-node positivity and consequently a poorer prognosis *(35).*

7.2. The Nuclear Localization Signal Sequence of HAP1

Despite the earlier discussion, in our hands transfection of the HAP1 cDNA into cultured human tumor-cell lines results in a predominantly nuclear localization of the protein. This has permitted us to define the amino acid residues in HAP1 that are required for targeting of the protein to the nucleus (unpublished results). We and others have noted the sequence similarity between the extreme N-terminal region of HAP1 and nuclear localization signal (NLS) sequences found in a variety of nuclear proteins. In HAP1, two basic clusters of residues, PKRGKK (residues 2–7) and KKSK (residues 24–27) fit the consensus for a bipartite NLS sequence. In order to confirm that these residues are important for nuclear targeting, we analyzed the effect of mutating or deleting residues within these motifs. Effects on nuclear targeting were detected by transfection of the modified cDNAs into HeLa cells followed by immunofluorescent detection of HAP1. Deletion of residues 1–36 prevents nuclear localization, confirming that the extreme N-terminal portion of HAP1 is vital for targeting of the protein to the nucleus. Mutation of lysines 24 and 25 in the KKSK motif also prevents nuclear localization, but mutation of all 4 basic residues in the PKRGKK motif does not, initially suggesting to us that the KKSK motif might represent the sole NLS sequence in HAP1. Nevertheless, this proved to be incorrect because a truncated HAP1 protein lacking residues 1–20, but retaining the KKSK motif, is not translocated to the nucleus. At this stage, the amino acids within this 20-residue N-terminal leader region that contribute to nuclear targeting are not defined, but may include the basic residues of the PKRGKK motif together with flanking residues.

8. REGULATION OF HAP1 GENE EXPRESSION

The *HAP1* mRNA is expressed constitutively at a relatively high level in many cell lines and tissues. Nevertheless, recent evidence indicates that certain cytotoxic agents can cause a modest increase in the level of HAP1 protein. This “induction” appears to be accompanied by an increase in cellular AP endonuclease activity. Ramana et al. *(49)* showed that *HAP1* mRNA peaks around 9–15 h after exposure of HeLa cells to

HOC1, and can also be induced by hydrogen peroxide, ionizing radiation, and bleomycin. This apparent response to different oxidants is abrogated by treatment of the cells with N-acetylcysteine, suggesting a role for ROS in the induction process. These authors also addressed whether the induction of HAP1 leads to protection against a subsequent challenge by a genotoxic agent (i.e., ROS an adaptive response). When analyzed 12 h after exposure to HOC1, HeLa cells showed an approx two-fold greater level of resistance to hydrogen peroxide, bleomycin, and MMS, but not to γ-rays or UV light. In a similar study using CHO cells Grösch et al. *(25)* showed that *HAP1* mRNA is induced by treatment with hydrogen peroxide and sodium hypochlorite. This induction of HAP1 is associated with an adaptive response to sodium hypochlorite in that it can confer some degree of resistance to a subsequent challenge by hydrogen peroxide. These authors also showed that transient transfection of the *HAP1* cDNA into CHO cells, which leads to an increase in cellular HAP1 levels, reduces the clastogenic effects of hydrogen peroxide. Consistent with *HAP1* being an oxidant-inducible gene, work by Fung et al. *(21)* showed that crocidolite asbestos induces HAP1 protein. This oxidant is important in the pathogenesis of mesothelioma in humans caused by exposure to asbestos.

Although the previous discussion seems to indicate that HAP1 is an inducible protein that confers an adaptive response to certain genotoxic agents, this effect has not been universally observed. For example, during their analysis of the promoter of the *HAP1* gene, Harrison et al. *(28)* failed to detect *HAP1* mRNA inducibility in HeLa cells by paraquat and bleomycin, among other oxidants. Moreover, Tomicic et al. *(65)* showed that overexpression of HAP1 in CHO cells does not confer resistance to MMS or hydrogen peroxide, apparently in contradiction to other published work from the same laboratory *(25,* cited earlier). Despite this, a recent paper by Herring et al. *(29)* showed that levels of HAP1 are associated with intrinsic radiosensitivity in cervical tumor cells, suggesting that the level of this enzyme may be important for cellular protection against x-rays in vivo.

Two studies have shown that hypoxic stress can stimulate expression of the HAP1 protein. Using HT29 cells Yao et al. *(81)* showed that *HAP1* mRNA is induced rapidly during hypoxic stress via a mechanism involving increased gene transcription. Moreover this leads to an accumulation of HAP1 protein that persists for several hours following return of cells to a normoxic environment. In our study, HAP1 protein was shown to be induced within 4 h of exposure to conditions of 1% oxygen and to peak at around 24 h *(66).*

One study has indicated that the HAP1 protein may regulate its own synthesis. During an analysis of HAP1 promoter activity, Izumi et al. *(32)* identified 2 nCARE-like sequences (negative Ca^{2+} response elements) that are responsible for inhibition of promoter activity. Mobility-shift assays showed that HAP1 protein itself binds to this nCARE sequence, suggesting that HAP1 may contribute to negative regulation of its own synthesis.

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5

Mating-Type Control of DNA Repair and Recombination in *Saccharomyces cerevisiae*

Jac A. Nickoloff and James E. Haber

1. INTRODUCTION

Mating type in the yeast *Saccharomyces cerevisiae* can be one of three types: **a**, α, and **a**α. These mating types reflect information present at *MAT,* which is normally *MAT***a** or *MAT*α in haploid cells, and *MAT***a**/*MAT*α in diploid cells. *MAT*α and *MAT* a each have two open reading frames, but functions have only been identified for three gene products, Mat**a**1p, Matα1p, and Matα2p. The *MAT* gene products are key regulators of the different stages of the yeast life cycle (reviewed in refs. *26,28,30*). The Mat**a**1p/Matα2p complex in diploid cells represses transcription of haploid-specific genes, including the repressor of meiosis, *RME1,* and *HO.* In *MAT*α haploid cells, Matα1p complexes with Mcm1p to activate α-specific genes. Matα2p complexes with Mcm1p (as well as Tup1p and Ssn6p) to repress a-specific genes. *MAT* controls mating: **a** cells mate with α cells but not with **a** cells, and vice versa, and **a**α cells do not mate with any of the three cell types. The mating behavior of **a** and α cells reflects their expression of the haploid-specific mating pheromones, **a**- and α-factor and the cognate transmembrane receptors. *MAT* also controls meiosis and sporulation; *MAT***a**/*MAT*α diploids can carry out meiosis whereas diploids expressing only one of the two alleles do not. The ability of **a**α diploids to enter meiosis depends on repression of the regulatory gene *RME11,* which is turned off by the action of the Mat**a**1p-Matα2p repressor complex. This leads to the expression of many meiosis-specific genes. Although natural **aa** or αα diploids are rare, they can be easily produced by using a regulated source of HO endonuclease *(29)* or by deleting one *MAT* allele (i.e. αΔ or aΔ) *(44).*

a and α information is also present at *HMR***a** and *HML*α, but in wild-type cells, these loci are silenced by the products of four *s*ilent *i*nformation *r*egulator *(SIR)* genes acting on sequences adjacent to *HMR***a** and *HML*α *(27,64).* Mating-type switching occurs in haploid mother cells following expression of HO endonuclease, which introduces a double-strand break (DSB) into *MAT,* thereby stimulating a gene-conversion event that transfers information from *HMR***a** or *HML*α to *MAT* (Fig. 1). Considerable effort has been directed toward developing molecular descriptions of the structure, expression, and silencing of mating-type loci, the functions of *MAT* gene products, the regulation of mating-type switching, and the recombinational mechanism that effects the switch *(26).*

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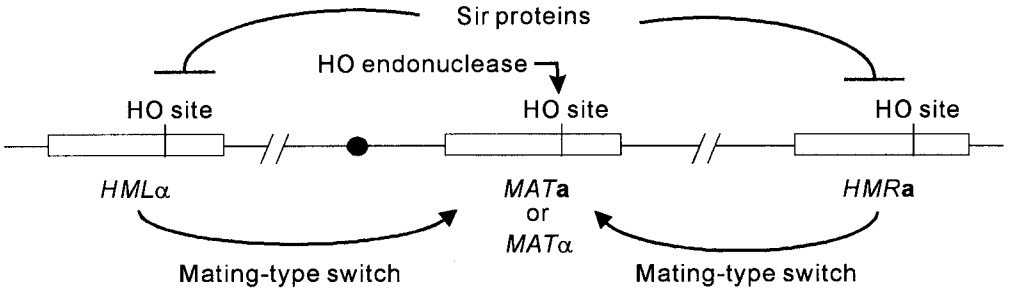


Fig. 1. Mating-type loci in yeast. *MAT* is an expressed locus on chromosome III and encodes either **a** or α information. Within the *MAT* locus is an HO recognition site; cleavage at this site stimulates gene conversion with the largely homologous *HML*α or *HMR***a** loci as donors, converting *MAT*α to *MAT***a** and vice versa. Sir proteins silence *HML*α and *HMR***a** and also block HO endonuclease cleavage at these loci.

This chapter focuses on *MAT* control of various aspects of general DNA repair and recombination, including radiation resistance, spontaneous and damage-induced homologous recombination, and nonhomologous end-joining (NHEJ). Another DNA repair process controlled by *MAT* is donor choice during mating-type switching; this topic was reviewed recently *(25)* and is discussed only briefly here.

2. DNA DOUBLE-STRAND BREAK REPAIR IN YEAST

DNA damage results from spontaneous chemical decomposition (e.g., deamination), chemical reactions of DNA with products of normal cell metabolism (e.g., oxidation), and exposures to exogenous agents including radiation and genotoxic chemicals. There are many types of DNA damage; each type can be classed as single- or double-strand damage. Single-strand damage, such as abasic sites, ultraviolet (UV)-induced pyrimidine dimers, and ionizing radiation (IR)-induced base damage can be repaired by base-excision and/or nucleotide-excision repair pathways, mediated in yeast by genes in the *RAD3* and *RAD6* epistasis groups. Single-strand damage also can be repaired by direct reversal, such as religation of single-strand breaks (SSBs), or photoreversal of pyrimidine dimers by photolyase. Although single-strand damage can stimulate recombination, this stimulation is not necessarily a consequence of the repair process. For example, UV-induced recombination is reduced when UV repair is enhanced, and there is evidence that single-strand damage stimulates recombination only after being converted to DSBs (*see* Chapter 13 and ref. *20*).

In yeast, the repair of double-strand damage (DSBs, interstrand crosslinks) often involves homologous recombination mediated by proteins encoded by genes in the *RAD52* epistasis group (reviewed in ref. *64,* and Chapter 16, Vol. 1). This group includes Rad51p, an *Escherichia coli* RecA homolog with strand exchange/pairing activities; Rad52p, a DNA end-binding protein; and Rad54p, an ATPase with putative helicase activity. Both Rad52p and Rad54p interact with Rad51p and stimulate the strand-exchange activity of Rad51p. Mammalian homologs have been identified for each of these proteins, and the interactions between them also appear to be conserved among eukaryotes (*see* Chapter 15). There are several distinct modes of homologous recombination, including conservative processes such as gene conversion and crossing

over, and the nonconservative process termed single-strand annealing (SSA) that operates between direct repeats. All of these events can result from DSB repair, with the particular outcome(s) dependent on the configuration of the recombining regions. An alternative mechanism of DSB repair is NHEJ, which can be imprecise and hence mutagenic, leading to deletions or insertions, or precise ("direct religation") when DSBs have cohesive, ligatable ends, such as those produced by nucleases. NHEJ is *RAD52*-independent, requiring *YKU70 (HDF1)* and *YKU80 (HDF2),* a special DNA ligase (DNA ligase 4), and its associated Xrcc4 protein (*LIG4* and *LIF1,* respectively, in *S. cerevisiae*) *(13).* yKu70p and yKu80p form the Ku heterodimer that has strong DNA end-binding activity. Yeast lacks the DNA-PKcs protein with which Ku proteins are associated in mammalian cells. Other genes that are important in NHEJ in budding yeast, but apparently not essential in either fission yeast or chicken DT40 cells include *RAD50, XRS2, MRE11 (88,95).* As with homologous recombination proteins, NHEJ proteins are conserved from lower to higher eukaryotes *(13).*

In mammalian cells, NHEJ is an important DSB repair mechanism involved in V(D)J joining and the repair of IR-induced DSBs. Because homologous recombination is so efficient in yeast, it was only recently recognized that NHEJ also has important roles in DNA repair. There are interesting differences between NHEJ in yeast and mammalian cells. For example, imprecise NHEJ is very rare in yeast *(41,55,61,71,72),* but quite common in mammalian cells *(47).* In mammalian cells NHEJ is effective for repairing both IR- and nuclease-induced DSBs since Ku mutants are sensitive to both types of DSBs (*see* Vol. 2, Chapter 17). In contrast, yeast *yku70* mutants have wild-type resistance to IR and nuclease-induced DSBs as long as homologous recombination is functional *(60,76).* Cells sensitive to IR generally show cross-sensitivity to the radiomimetic agents methylmethane sulfonate (MMS) and bleomycin. It is interesting that haploid yeast Rad^+ Ku^- strains are resistant to IR, but show mild sensitivity to MMS *(56,60)* and marked sensitivity to bleomycin *(53).* Apparently, a fraction of MMS and bleomycin damage cannot be repaired by homologous recombination in haploid cells, but instead is repaired by a Ku-dependent (NHEJ?) pathway. In contrast to the haploid MMS results, a diploid Ku^- strain showed no MMS sensitivity *(76),* consistent with enhanced homologous recombination in diploids (*see* Subheading 3.1. and 3.7.). Even in a *rad52* background where homologous recombination is effectively absent, *yku70* mutation confers only slightly more IR sensitivity, indicating that IR-induced DSBs are rarely processed by NHEJ in yeast *(76).* In contrast, nuclease-induced DSBs are efficiently repaired by (precise) NHEJ (*see* Subheading 3.6.). Thus in yeast, homologous recombination and NHEJ compete for repair of nuclease-induced DSBs, but most or all IR-induced DSBs are processed by homologous recombination. This accounts for the marked IR sensitivity of G1 haploids as these lack homologous repair templates (*see* Subheading 3.1.). The different repair efficiencies for nuclease- and IR-induced DSBs by NHEJ lead to interesting differences in the effects of *MAT* on homologous recombination stimulated by these different types of DSBs, as discussed in Subheading 3.

3. *MAT* CONTROL OF RADIATION RESISTANCE AND RECOMBINATION

3.1. Distinction between Effects of Ploidy and MAT Heterozygosity on Radiation Resistance

It was established very early that diploid yeast cells are more resistant to the cytotoxic effects of IR than haploid cells *(43).* Similarly, haploid G2 cells are more radiore-

sistant than G1 cells *(11,68).* These results could reflect the availability of homologous chromosomes or sister chromatids that act as repair templates for homologous recombination. Although this is a reasonable idea, it was recognized early that heterozygosity at *MAT* was responsible for at least part of the increased resistance because **a**/α diploids were more radioresistant to IR than **aa** or αα diploids *(62).* The higher IR resistance of **a**α diploids was confirmed in many subsequent studies *(17,21,31,34,36,51,70,73).* The effect of *MAT* heterozygosity is seen in both G1 and G2 cells, but more marked effects are seen in G1 *(36).* Although radioresistance generally increases with increasing DNA content, tetraploids are actually less radioresistant than diploids *(36,62).* In contrast to the diploid results, *MAT* heterozygosity in haploid cells does not increase radioresistance *(42),* suggesting that the diploid *MAT* effects reflect enhanced homologous recombination that is not possible in haploids (i.e., in G1 cells). Haploid strains are normally homozygous at *MAT* but can be made heterozygous by introducing a second copy of *MAT* on a circular plasmid, or by activating *HML*α and *HMR***a** by disabling a *SIR* gene. Together these results clearly indicate that resistance to IR is influenced independently by ploidy and *MAT* genotype.

Saeki et al. *(70)* connected the radioprotective effect of *MAT* heterozygosity to DSB repair and recombination by showing that mutations in genes involved in recombinational repair *(RAD51, RAD52,* and *RAD54)* abolished the radioprotective effect. Two other important recombination genes are *RAD55* and *RAD57,* which encode proteins of a heterodimer *(35,74,79).* Interestingly, deletions of these genes cause radiosensitivity only at low temperature and this defect can be suppressed by overexpression of *RAD51 (35),* and by *MAT* heterozygosity *(21,51).* In contrast, cells defective in either excision repair (*RAD3* epistasis group) or error-prone repair (*RAD6* epistasis group) still display greater radioresistance when *MAT* is heterozygous *(31,70).* The idea that *MAT* heterozygosity influences radioresistance through effects on homologous recombination was further supported by the finding that the radioresistant phenotype of *rad52-20* mutant cells was suppressed by heterozygosity at *MAT* (as well as by overexpression of *RAD51*). *rad52-20* haploids and *MAT* homozygous diploids show marked sensitivity to IR, but haploid *rad52-20 sir* double mutants, and *MAT* heterozygous diploids display essentially wild-type radioresistance *(73).* Thus, the protective effects of *MAT* heterozygosity to IR damage reflect, at least in part, enhanced homologous recombination (*see* Subheadings 3.5. and 3.7.).

3.2. Effects of MAT on Resistance to UV and MMS

In contrast to the results with IR, several studies showed that *MAT* status had little or no effect on UV resistance in Rad$^+$ cells *(18,21,50).* However, in mutants sensitive to killing by UV, *MAT* heterozygotes were markedly more resistant to killing by UV than *MAT* homozygotes. In fact, some mutants defective in excision or error-prone repair pathways, such as *rad18,* display even stronger *MAT* effects on UV resistance than those seen in Rad$^+$ cells with IR. These results can be explained by the idea that some damage normally processed by the UV repair pathways can be channeled into the recombinational repair pathway; see Heude and Fabre *(31)* and references therein. It was also shown that the effects of *MAT* heterozygosity on cell survival following DNA damage reflect functions of both the Mat**a**l and Matα2 gene products, but were independent of *RME1,* a known downstream target of the Mat**a**1/Matα2 repressor. To date, the specific

target(s) of this repressor complex responsible for the effects of *MAT* on radioresistance have not been identified. In contrast to UV but similar to IR, *MAT* heterozygotes are more resistant than homozygotes to the cytotoxic effects of alkylating agent MMS *(50).* This is not surprising because both MMS and IR produce SSBs and DSBs.

3.3. Effects of MAT on UV-Induced and Spontaneous Homologous Recombination

It is not known how spontaneous recombination is initiated, but DSBs are often suggested as possible initiators. Spontaneous DSBs might arise at stalled replication forks *(69),* replication past SSBs (*see* Chapter 2 and ref. *24*), and in a cell cycle-independent manner from repair of oxidative or other forms of spontaneous DNA damage. A central role in vertebrate cells for RAD51-mediated recombination in replication restart at stalled or collapsed replication forks or in lesion bypass is suggested by the finding that RAD51 is essential for viability of higher eukaryotic cells, and that cells depleted for RAD51 accumulate chromosome and chromatid breaks *(48,77).* DSBs also may initiate UV-induced recombination, although much of this evidence is indirect (*see* Chapter 13).

Friis and Roman *(18)* were the first to show that *MAT* heterozygotes had higher homologous recombination frequencies than *MAT* homozygotes. In this study, *MAT* heterozygosity increased UV-induced allelic (homolog) recombination by three-fold. Similar *MAT* effects on UV-induced recombination were obtained in subsequent studies with allelic *(14,21)* and ectopic recombination substrates *(16).* As described for the radioprotective effects of *MAT,* enhanced recombination in *MAT* heterozygotes is clearly owing to *MAT,* and not just ploidy, because enhanced recombination is seen both in diploids and in haploids expressing both **a** and α *(16).* One concern of recombination studies is that *MAT* heterozygotes have the capacity to sporulate and some cells do so even on rich medium. In this small fraction of cells recombination would be increased to meiotic levels, and this could give an apparent increase in mitotic recombination. In most studies meiosis is thought to contribute little or not at all to the observed differences; this question could be answered by using *spo11* mutants, which fail to induce meiotic DSBs but are otherwise recombination-competent *(37).*

Three studies showed that spontaneous allelic recombination was higher in *MAT* heterozygotes than homozygotes *(15,21,36).* However, *MAT* genotype did not affect spontaneous sister chromatid recombination *(36),* suggesting that *MAT* heterozygosity may only enhance interactions between homologs. The lack of a *MAT* effect on sister chromatid recombination may be a consequence of the close, topologically constrained association of sister chromatids *(23,33),* such that *MAT* heterozygosity does not further stimulate these interactions. This view is consistent with the finding that sister chromatids are preferred recombinational repair templates in yeast *(36).* An alternative, but not mutually exclusive idea is that *MAT* genotype directly controls the frequency of spontaneous allelic recombination, perhaps by modulating the levels of recombination proteins. One such protein is Tid1p (Rdh54p), which is a homolog of Rad54p. Deletion of this protein reduces interchromosomal but not intrachromosomal homologous recombination *(3,40,75).* Tid1p plays a much more central role in meiosis, where it interacts with meiosis-specific strand-exchange protein, Dmc1p (a Rad51p homolog). Curiously, Tid1p is expressed at higher levels in **aa** and αα cells than in **a**α cells *(19).*

It is possible that the effects of *MAT* on homologous recombination and cell survival reflect specific effects on DSB repair, including recombinational repair. Although this idea is appealing in its simplicity, it will be very difficult to rule out other factors (e.g., changes in chromatin structure), because a large number of genes, many with unknown functions, are differentially expressed in the different mating types *(19,94).*

3.4. Recombination Between Ty Elements is Not Influenced by MAT

Studies of Ty recombination provide some interesting contrasts with other recombination systems. IR does not enhance Ty recombination involving direct repeat popouts, or conversions of a marked (target) Ty by any of the approx 30 unmarked Ty elements scattered throughout the genome, regardless of *MAT* status *(42).* Thus, *MAT* heterozygosity does not have global effects on homologous recombination. It is noteworthy that Ty elements are generally refractory to damage-induced recombination—even UV, a very potent recombinogen, has minimal stimulatory effects (and no *MAT* effects) on ectopic Ty recombination *(42).* In the same cells used to measure Ty recombination, ectopic recombination between 300 bp direct repeats at a non-Ty locus was monitored simultaneously, and modest enhancements with IR were observed. In agreement with Kadyk and Hartwell *(36)* but not Fasullo and Dave *(16), MAT* had no effect on the frequency of these non-Ty events *(42).* It is unclear why recombination in Ty elements is poorly enhanced by UV and IR, and not influenced by *MAT,* but it is clear that Ty elements are able to recombine, because DSBs introduced into a Ty element by HO endonuclease strongly enhance Ty recombination *(65).* Although not yet tested, it seems likely that the frequency of HO-induced Ty recombination events will be affected by *MAT* owing to *MAT* control of precise NHEJ (*see* Subheading 3.6.).

3.5. Effects of MAT on Ionizing Radiation- and HO-Induced Homologous Recombination

Although DSBs are potent initiators of homologous recombination (reviewed in refs. *63,64*), and IR is very effective at producing DSBs *(84),* recombination is only moderately enhanced by IR. This is because DSBs are most effective at stimulating recombination when they occur within or near a target gene, and each irradiated cell is likely to sustain considerable damage at other loci before a target gene is damaged in a significant fraction of a cell population. The effects of *MAT* on IR-induced recombination have been variable (Table 1, studies 1–4). *MAT* genotype had no effect on X-ray-induced recombination: unequal sister chromatid recombination between short (305 bp) direct repeats was not stimulated by X-rays in **aa** or **a**α cells, and both cells types showed the same increases in allelic recombination at each dose tested *(36).* In contrast, *MAT* heterozygosity increased by about two-fold the level of IR-induced ectopic recombination between duplicated regions of *his3* (~500 bp in length) located on different chromosomes; in this system selected His^+ recombinants reflect only reciprocal chromosome translocations *(17).* Interestingly, although *MAT* influenced IR-induced *his3* translocations in diploids, introduction of a plasmid carrying the opposite *MAT* allele had no effect on these same *his3* substrates in haploids *(17).* It was suggested that this ploidy difference reflected stimulation in diploids by damage sustained by homologous chromosomes not carrying the translocation substrates, by so-called triparental recombination events *(67);* such interactions are not possible in haploids. In contrast to the results

Table 1
***MAT* Effects on IR- and HO-Induced Recombination**

Study	Ploidy	Recombination substrate	Damaging agent	Recombination		Reference
				Stimulation	Increased by *MAT* heterozygosity?	
1	Diploid	Sister chromatid	Ionizing radiation	None	No	*(36)*
2	Diploid	Translocation	Ionizing radiation	Moderate	Yes	*(16)*
3	Haploid	Translocation	Ionizing radiation	Low	No	*(16)*
4	Diploid	Allelic	Ionizing radiation	Moderate	No	*(36)*
5	Diploid	Translocation	HO endonuclease	Strong	Yes	*(16)*
6	Haploid	Translocation	HO endonuclease	Strong	Yes	*(16)*
7	Diploid	Allelic	HO endonuclease	Strong	Yes	*(44)*
8	Diploid	Allelic	HO endonuclease	Strong	Yes	*(38)*

with IR, studies of HO-induced recombination show a much more consistent picture, with strong enhancements of ectopic (translocation) and allelic events, and marked effects of *MAT* heterozygosity (Table 1, studies 5–8). It is particularly interesting that damage-induced translocations show no effect of *MAT* heterozygosity with IR, but clear effects with HO endonuclease.

How can these disparate results be reconciled? To answer this, we need to draw on several lines of evidence. First, inactivation of NHEJ, by *yku70* or *yku80* knock-out, does not decrease radioresistance in yeast *(60).* This contrasts with marked radiosensitivity conferred by Ku mutations in mammalian cells (*see* Chapter 17 in Vol. 2). Furthermore, a *yku70 rad52* double mutant is only slightly more radiosensitive than a *rad52* single mutant *(76).* Thus, NHEJ is not involved to any great extent in the repair of IR damage in yeast. Second, *MAT* heterozygosity increases resistance to IR, an effect that is eliminated by mutations in recombinational repair genes (*RAD52* epistasis group). Together, these results indicate that homologous recombination is by far the most important repair pathway for IR damage, and that *MAT* heterozygosity increases radioresistance by enhancing recombinational repair, perhaps by enhancing pairing activity.

The magnitude of any effect of *MAT* on pairing is likely to depend on the intrinsic pairing properties of the recombination substrate under study. For example, sister chromatid recombination may not be enhanced by *MAT* heterozygosity (Table 1, study 1) because of the close association between sister chromatids, as discussed in Subheading 3.3. In contrast, the limited pairing between ectopic repeats on nonhomologous chromosomes may be enhanced by *MAT* heterozygosity (Table 1, study 2). However, because this effect was seen in diploids but not haploids (Table 1, compare studies 2 and 3), enhanced pairing may be limited to homologs (revealed as triparental recombination events). From this discussion, one would predict that IR-induced allelic interactions would be enhanced by *MAT* heterozygosity, but this was not the case (Table 1, study 4). This may reflect selection bias since a *MAT*-dependent increase in recombination events might go undetected if a large fraction of events involved co-conversion of the *leu1-1* and *leu1-12* alleles examined in this study (producing unselected homozygous *leu1-1/leu1-1* or *leu1-12/leu1-12* products). To understand the effects of *MAT* on HO-induced events and how these differ from IR-induced events, we need to consider the difference between precise and imprecise NHEJ (discussed in the next section), and the effects of *MAT* on NHEJ (discussed in Subheading 3.7.).

Finally, there is likely to be an inherent difference between the way nuclease-cleaved DNA engages in recombination compared to IR-broken molecules. One indication of this difference emerges from examining the effect of a single DSB on the viability of cells deleted for the *RAD52* recombination gene. Ho and Mortimer *(32)* reported that a single X-ray-induced DSB was dominant lethal in *rad52* haploids, diploids and even tetraploids, but this is clearly not the case in *rad52* diploids suffering an HO-induced DSB *(41,54).* IR-induced DSBs likely have glycol fragments attached to the phosphates and may be channeled into somewhat different recombination repair pathways.

3.6. Imprecise and Precise NHEJ in Yeast

Because DSB-repair in yeast by *RAD52*-dependent homologous recombination is so efficient, early strategies to detect NHEJ in yeast chromosomes employed *rad52*

mutants *(41,61),* or systems in which DSBs were introduced into regions for which no homologous repair template was available *(55,71,72).* The repair of HO endonuclease breaks and dicentric chromosome breaks by NHEJ results in similar junctions *(41).* These selective systems detected only imprecise NHEJ events, which were quite rare (typically <1% of homologous recombination levels). A nonselective assay was used to monitor imprecise NHEJ in recombination-competent cells (i.e., Rad^+ with DSBs introduced into a region of shared homology). Imprecise NHEJ of HO-induced DSBs was shown to occur at ~0.1% of homologous recombination levels *(38),* similar to *rad52* results. Thus, the efficiency of imprecise NHEJ is independent of Rad52p.

The identification of yeast homologs of mammalian NHEJ genes, Ku70 and Ku80 *(9,10,60),* and the use of a plasmid transformation-based rejoining assay *(9,10,60,83),* greatly facilitated studies of NHEJ in yeast. In particular, these assays indicated that nuclease DSBs in plasmid DNA are repaired very efficiently by precise NHEJ, and that this repair requires *YKU70, YKU80, LIF1,* and *LIG4,* and also involves *RAD50, XRS2, MRE11* (reviewed in ref. *13*).

It has been difficult to measure the efficiency of precise NHEJ of chromosomal DSBs because such events are not easily distinguished from "nonevents" (i.e., no DSB). However, the fact that yeast cells are able to survive expression of *Eco*RI, which has thousands of target sites per genome strongly suggested that precise NHEJ of nuclease DSBs in chromosomal DNA was quite efficient *(5,45,46).* A more quantitative measure of precise NHEJ of nuclease DSBs in chromosomal DNA was provided by Lee et al. *(44).* In this study, HO cleavage of *MAT* in haploid cells deleted for *HML* and *HMR* is largely lethal. However, when HO expression was limited to 1 h, 33% of cells survived compared to <1% survival of a *yku70* mutant. These results indicate that at least 33% of HO-induced chromosomal DSBs can be repaired by precise NHEJ. A different approach led to a similar conclusion in haploid cells, with at least 20% of HO DSBs in chromosomal DNA repaired by precise NHEJ (*see* Subheading 3.7.). Precise repair of nuclease-induced DSBs in mammalian chromosomes was recently reported *(49).*

3.7. Mating-Type Regulation of NHEJ and Homologous Recombination

About the same time that Ku was shown to mediate NHEJ in yeast, other lines of investigation revealed numerous connections between Ku and telomere structure/function (reviewed in refs. *27,52*). *yku 70* and *yku80* mutants were found to have shortened telomeres, and a similar phenotype was seen in *tel1* mutants *(9,66).* Genes near telomeres are silenced by binding of Sir2p, Sir3p, and Sir4p to telomere sequences; these proteins also silence *HML* and *HMR.* Extra DNA termini were found to relieve telomeric silencing *(87),* suggesting that one or more factors competed by DNA termini was involved in silencing, such as Ku, with its known end-binding function, or silencing proteins such as Sir, Rap1p, or Rif proteins. The involvement of Ku in telomere structure and function raised the possibility that other telomere-associated proteins (i.e., Sir2-4p and Tel1p) might be involved in NHEJ, and experiments appeared to confirm this possibility: NHEJ in yeast (assayed by plasmid end-rejoining) was reduced by 10- to 20-fold in *sir2, sir3* or *sir4* mutants, but not in a *tel1* mutant *(8,82).* Furthermore, *sir2, sir3,* and *sir4* mutants each displayed radiosensitivity phenotypes remarkably similar to *yku70* mutants, with increased sensitivity to IR revealed only in a *rad52* mutant background *(82).*

Initially these results were interpreted to mean that Sir proteins were directly involved in NHEJ. However, two recent studies showed that most or all of the *sir* effect on NHEJ is owing to *MAT* heterozygosity. In a study using transfected linear plasmids, deletion of all three mating-type loci suppressed the *sir*-dependent reduction in plasmid end-rejoining *(4).* In the second study *(44),* NHEJ was monitored for DSBs induced in vivo in plasmid and chromosomal DNA. In a *MAT***a** haploid lacking *HML*α, *sir* mutants do not yield an **a**α state and *sir* mutants were shown to have wild-type levels of plasmid retention (~50%) when DSBs were created by nucleases or by mechanical breakage of a dicentric plasmid. In contrast, plasmid retention was strongly reduced in *yku70* mutants. Similar results were obtained for chromosomal DSBs. In haploid wild-type cells deleted for *HML* and *HMR,* continuous expression of HO (which cleaves *MAT*α) was largely lethal because homologous recombination is blocked (~0.1% survivors). Survivors arise by NHEJ that yields deletions or insertions in *MAT*α (preventing further cleavage by HO). Survival is reduced an additional 3-orders of magnitude in *yku70,* demonstrating the importance of Ku for these imprecise NHEJ events, but *sir2* had only a modest two- to three-fold effect on survival. Thus, the effect of *sir2* on NHEJ is largely owing to *MAT* heterozygosity. The chromosomal assay (but not the plasmid rejoining assay) revealed that *sir3* and *sir4* mutants had two-fold lower levels of NHEJ regardless of *MAT* genotype (*sir2* had a smaller effect) *(44).* These results suggest that Sir proteins also have a direct role in end-joining, and this has been supported by cytological and chromatin immunoprecipitation experiments, indicating that both Sir and Ku proteins translocate from telomeres to sites of DSB damage *(56,57,59).* It is not known how *MAT* controls NHEJ. Expression levels of several genes involved in NHEJ were measured in *MAT* homozygous and heterozygous strains, including *YKU70, YKU80, LIG4, XRS2,* and *MRE11,* but none were differentially expressed *(4);* however, the use of microarray technology has revealed other genes that are downregulated by mating type and that might be involved in NHEJ *(19).*

Because downregulation of NHEJ by *MAT* heterozygosity serves to increase HO-induced homologous recombination, one would predict that elimination of NHEJ by *yku70* mutation would similarly increase recombination. In agreement with this prediction, recombination induced by HO endonuclease cleavage of a chromosomal *ura3* direct repeat substrate in haploid cells was 1.25-fold higher in a *yku70* mutant than wild-type *(38).* This result suggests that at least 20% of HO-induced DSBs are repaired by precise NHEJ in wild-type haploid cells. The opposite result was seen for HO-induced mating-type switching, with *yku70* mutants displaying three-fold lower levels than wild-type; *yku70* mutants also had 10- to 40-fold lower levels of spontaneous allelic recombination *(53).* However, in this study the W303-derived cells likely carried a *rad5* mutation, and the reduced recombination in *yku70* may reflect a genetic interaction with *rad5.* This idea is supported by a study showing that Rad5 is involved in channeling repair from NHEJ to gene conversion *(1).* In another study it was found that *yku70* mutants have wild-type levels of meiotic recombination *(83).* The lack of an effect of *yku70* on meiotic recombination is consistent with a reduced (or minimal) role for NHEJ in **a**α cells (the required *MAT* genotype for meiosis). Interestingly, Ku is reduced in abundance in mammalian meiotic cells *(22);* this reduction may be required to minimize NHEJ in higher eukaryotic cells during meiosis, thereby promoting homologous recombination.

It is now clear that the NHEJ pathway in yeast is ineffective in the repair of IR damage, very effective at precise rejoining of nuclease-induced (ligatable) DSBs, upregulated in cells homozygous at *MAT,* and downregulated in cells heterozygous at *MAT.* In this light, the differential effects of *MAT* heterozygosity on IR and HO-induced homologous recombination shown in Table 1 can be readily explained. Both homologous recombination and NHEJ compete for the repair of HO-induced DSBs. In *MAT* homozygous strains, NHEJ is upregulated and this reduces the number of DSBs processed by homologous recombination. In *MAT* heterozygous strains, NHEJ is downregulated and homologous recombination increases because essentially all DSBs are shunted to the homologous recombination pathway. Thus, *MAT* indirectly influences nuclease-induced recombination by modulating NHEJ. As described in Subheadings 3.1. and 3.5., *MAT* directly influences IR-induced recombination. There is evidence that *MAT* heterozygosity also enhances nuclease-induced homologous recombination, independently of its effects on NHEJ. A study of HO-induced allelic recombination vs chromosome loss showed that recombination was nearly 100% efficient in an **a**α diploid but only 82% efficient in an Δα diploid *(44).* Similarly, even when NHEJ is inactivated by *yku70* mutation, HO-induced allelic recombination was higher in **a**α cells than in **aa** cells *(38),* although an alternative explanation for this result is that DNA ends in *yku70* mutants might be altered (i.e., longer single-stranded tails) and might be better substrates for a *MAT*-regulated protein involved in allelic recombination, such as Rdh54p *(40,75)* which is expressed at five-fold higher levels in **aa** cells than in **a**α cells *(19).*

It has been proposed that upregulation of NHEJ in *MAT* homozygous strains reflects an increased need for this type of repair in G1 haploid cells as these lack homologous repair templates *(4,44).* This hypothesis suggests that homologous recombination is the preferred repair mode when available. Recombination has been suggested to confer both short- and long-term (evolutionary) advantages. Recombination is an essential feature of meiosis, with crossovers required for accurate chromosome segregation *(12),* and both meiosis and *MAT* heterozygosity confer competitive growth advantages in mixed culture experiments *(7).* An alternative, but not mutually exclusive hypothesis derives from considerations of the types of DSBs formed most frequently in natural settings, including those that result from replication, mechanical stress (i.e., in dicentric chromosomes), endogenous nucleases (including failed type II topoisomerase reactions), or other DNA repair processes such as nucleotide-excision repair operating on closely opposed pyrimidine dimers. For perhaps many of these types of DSBs, even if NHEJ were possible, repair by this pathway might not be precise. It may be that high-fidelity repair by homologous recombination, despite the potential for gross chromosomal rearrangement, provides for optimal genome stability.

4. MATING-TYPE REGULATION OF DONOR PREFERENCE DURING HO-INDUCED SWITCHING OF THE *MAT* LOCUS

One of the most fascinating aspects of mating-type control of recombination is that during HO-induced switching of the *MAT* locus, *MAT***a** and *MAT*α cells show a strong preference to interact with different silent mating type donors, *HML* and *HMR (39).* Thus, a *MAT***a** cell will recombine with *HML* four to five times more often than with *HMR.* This preference persists even if both *HML* and *HMR* carry the same Y**a** or Yα region and even if *HML* is replaced with a cloned *HMR* segment, indicating that chro-

mosomal position dictates this preference *(85,92).* In fact, recombination is regulated along the entire 113 kbp left arm of chromosome III, as an *HML* or *HMR* sequence placed at several other locations along this arm is preferentially selected in *MAT***a** cells and preferentially excluded in *MAT*α cells *(90,91,93).* Moreover, the activation of the left arm for recombination (or rather, the repression of the left arm in *MAT*α cells) is not specific to HO-induced events involving silent donor sequences; a *leu2-R* allele inserted into a complete deletion of *HML* and its surrounding silencer sequences shows higher levels of recombination with a *leu2-K* allele in *MAT***a** vs *MAT*α cells, even when the *leu2-K* allele is present on another chromosome. Evidence suggests that in *MAT*α cells the left arm is somehow made inaccessible for recombination, as a *MAT*α cell carrying *HML*α but deleted for *HMR* frequently dies rather than completing recombination using the (excluded) left-arm donor *(90).* Whatever accounts for this exclusion, it is not a general heterochromatinization or condensation of the left arm, as there is no evident change in the expression of genes along the arm in *MAT***a** versus *MAT*α cells, as measured by microarrays *(19).*

A major breakthrough in understanding the mechanism of donor preference was the identification of a small *cis*-acting sequence that controls donor preference, located about 17 kbp centromere-proximal from *HML (91).* This Recombination Enhancer (RE) was "whittled down" to a 244 bp sequence that retains much of the activity *(89).* Further dissection has indicated that three subregions comprising only 137 bp has activity (K. Sun and J.E.H., unpublished). When RE is deleted in *MAT***a** cells, the left arm adopts the "cold" phenotype found in *MAT*α cells. Further work showed that the inactivation of RE was accomplished by the binding of the Matα2p-Mcm1p co-repressor, which is known to regulate **a**-specific genes *(80,81,89).* Under these conditions, the entire 2.5 kbp noncoding region in which RE resides is occupied by highly positioned nucleosomes. Activation of RE in *MAT***a** cells depends on the Mcmlp protein, which also activates transcription of **a**-specific genes, although the roles of other proteins that presumably bind to the other segments of RE have not been identified. How RE acts to antagonize the coldness of the "default" state remains a mystery. One general idea is that the left arm is sequestered—perhaps bound to the nuclear envelope—in *MAT*α cells, thus preventing easy diffusion to participate in recombination, but is somehow freed in *MAT***a** cells.

5. CONCLUDING REMARKS

There are several key questions that remain about *MAT* regulation of DNA repair and recombination. A major gap in our knowledge concerns the mechanism by which *MAT* regulates NHEJ. It is unclear whether *MAT* effects on NHEJ and homologous recombination are genetically separable, and whether they reflect pleiotropic effects of a single gene or set of genes. Microarray analysis of mRNA levels in cells with various *MAT* genotypes has provided a huge number of candidate targets for further investigation *(19).* However, *MAT* control of NHEJ may reflect post-transcriptional regulation and may not be revealed by analysis of mRNA levels. The mechanism of action of RE also remains a mystery. A molecular description of the RE regulatory system should provide important insights into mechanisms of recombination suppression in yeast and higher eukaryotes as well.

It is now apparent that organisms regulate recombination and DNA repair in many ways, during different stages of the cell cycle, during different developmental and life-

cycle stages, and in response to environmental insults. Even within a cell, recombination is differentially regulated at various chromosomal domains. The *MAT* locus represents a particularly interesting case because it has strong regulatory effects on a variety of recombination and repair processes, and *MAT* expression is itself regulated by a recombinational repair event. Metazoans also employ recombinational repair to control gene expression, exemplified by gene rearrangements during development of the immune system (*see* Chapter 11). It is likely that higher eukaryotes employ a variety of mechanisms by which cellular recombination and repair capacities are regulated during growth and development. The p53 protein provides one such example: embryonic stem cells and differentiated somatic cells differ markedly in their p53 responses to DNA damage, including altered translocation of p53 to the nuclease, altered checkpoint function, and altered apoptotic response *(2).* Wild-type p53 functions to repress homologous recombination *(6,58,78,86)* and embryonic stem cells display enhanced homologous recombination (as measured by gene-targeting efficiency). p53 repression of homologous recombination may be transiently relieved in embryonic stem cells to enhance recombinational repair of endogenous lesions during the characteristic rapid growth of these cells. The elucidation of this and other networks that modulate DNA repair and recombination in different cell types and developmental states will provide many interesting challenges for the future.

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6

DNA End-Processing and Heteroduplex DNA Formation During Recombinational Repair of DNA Double-Strand Breaks

Galina Petukhova, Eva Y.-H. P. Lee, and Patrick Sung

1. INTRODUCTION

1.1. Pathways of DNA Double-Strand Break Repair in Eukaryotes

Two enzymatic mechanisms, DNA end-joining and homologous recombination, operate in eukaryotic cells to repair DNA double-strand breaks (DSB) induced by ionizing radiation and by other agents. The recombinational repair pathway relies on an intact DNA homolog to direct the healing of the DNA break, and is designed to restore the original configuration of the injured chromosome. In contrast, the DNA end-joining process has no requirement for a DNA homolog and often results in gain or loss of genetic information, and at times chromosomal rearrangements and translocations. There is emerging evidence that DNA end-joining and homologous recombination are differentially required at specific stages of the cell cycle, with the former appearing to be the more critical mechanism in the G1 phase and the latter taking on a prominent role in late S and G2 when a sister chromatid becomes available to direct the repair process *(113)*. In this article, we will provide an account of what is currently known about homologous recombination and its DNA repair role.

1.1. Repair by Homologous Recombination

This is an excellent time to review DNA DSB repair by homologous recombination. A great many years of exhaustive genetic studies in *Saccharomyces cerevisiae* have served up conceptual and genetic frameworks for appreciating the intricacies of recombination processes and, most importantly, have paved the path for mechanistic dissection of these processes utilizing biochemical and cell biological approaches. The recent identification of genes in higher eukaryotes that are clear analogs of the yeast recombination/repair genes further indicates that studies in *S. cerevisiae* will be informative with respect to these processes in mammals. Nonetheless, although there is little doubt that the fundamental mechanisms of recombination processes have been conserved from yeast to humans, these processes in higher eukaryotes are apparently subject to additional layers of control, some of which are dependent on known tumor suppressors

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including the gene mutated in ataxia talengiectasia *(ATM),* the breast tumor-suppressor genes *BRCA1* and *BRCA2,* the gene mutated in Nijmegen breakage syndrome *(NBS),* and almost certainly with additional as yet unknown genes. Although the precise manner in which these tumor-suppressor genes modulate the DNA DSB repair process remains to be elucidated, these observations, coupled with the known role of recombination genes in helping maintain genomic stability, have provided tantalizing clues as well as tangible evidence that the recombination machinery is intimately linked to cancer suppression in mammals.

1.3. The RAD52 Epistasis Group Required for Recombination Processes

Genetic experiments in yeast have indicated that aside from its DNA repair role, the recombination machinery functions to establish stable interactions between chromosomal homologs during the meiotic prophase, and as such, is indispensable for the proper disjunction of homologs in meiosis I *(91).* In *S. cerevisiae,* the two meiotic divisions, if successfully completed, yield four haploid spores. The requirement for homologous recombination in meiotic chromosome homeostasis is aptly manifest as a nearly complete failure of recombination defective mutants to undergo sporulation, and low viability of the few spores that are formed *(91).* In addition, the process of mating-type switching in *S. cerevisiae,* which is initiated by a site-specific DNA DSB at the mating-type locus made by the HO endonuclease, is also dependent on the recombination functions for its completion *(77).* Much of the present knowledge about recombination in *S. cerevisiae* has in fact been garnered from studies on meiotic recombination, HO-induced mating-type switching, and from other model systems involving HO-induced recombination processes.

A number of genes, namely, *RAD50, RAD51, RAD52, RAD54, RAD55, RAD57, RAD59, RDH54/TID1, MRE11,* and *XRS2,* collectively known as the *RAD52* epistasis group, are required for homologous recombination processes *(81,35,4,55,102).* Aside from the *RAD52* epistasis group, the meiotic recombination program is dependent on a plethora of meiosis-specific factors, some of which are involved in the introduction of DNA DSB at various recombination "hotspots" along each of the chromosomes (*see 54* for a discussion), while others function in checkpoint mechanisms coordinating recombination and cell-cycle progression *(64,123),* and the *RAD51* homolog *DMC1 (10,11).*

1.4. DNA Double-Strand Breaks as Progenitor of Homologous Recombination

Because DNA DSBs are potentially lethal lesions, intuitively, it is somewhat curious as to why programmed recombination processes are initiated via the formation of these breaks. This curiosity aside, it is a very well-established fact that mating-type switching and meiotic recombination in *S. cerevisiae* are triggered by the introduction of DNA DSBs at discrete chromosomal loci. Genetic experiments have provided evidence that the meiotic DNA DSBs are made by a multicomponent complex comprising Rad50, Mre11, Xrs2, Spo11, and other proteins. Interestingly, Spo11 shows significant sequence homology to an archaebacterial type II topoisomerase, and mutation of tyrosine 135 in Spo11, which is equivalent to the active site tyrosine in other topoisomerases, abolishes its biological function *(9).* These observations, together with the fact that Spo11 can be found associated with the 5′ termini of the ends of the DNA breaks *(54),* lend credence to the suggestion that Spo11 protein is the catalytic subunit of the protein machinery that makes DNA DSBs during meiotic prophase *(9,54).*

Importantly, recent studies have identified structural and functional homologs of Spo11 in other eukaryotes. Villenenue and colleagues *(25)* have reported that inactivation of the Spo11 homolog in *Caenorhabditis elegans* results in no overt somatic abnormalities but a constellation of phenotypes consistent with a defect in meiotic chromosome segregation. As assessed by cytological and genetic methods, meiotic crossovers are abolished in worms lacking the Spo11 protein. Mutant *spo11* worms produce a normal number of oocytes, but the vast majority die as embryos. Of the few mutant embryos that survive to adulthood, roughly half are male (XO). As *C. elegans* is normally hermaphroditic possessing two X chromosomes, the preponderance of XO males is an indication of nondisjunction of the X chromosomes. The suggestion that the meiotic defects in the *spo11* mutant animals are owing to an inability to form DNA DSBs is reinforced by the finding that meiotic crossovers and embryonic viability can be restored by treatment of the *spo11* mutant worms with γ-rays. Taken together, the results strongly suggest that like its yeast counterpart, *C. elegans* Spo11 is also required for meiotic recombination, probably for the introduction of meiosis-specific DNA DSBs *(25).* The *mei-W68* gene in the fruitfly *Drosophila melanogaster* is required for meiotic gene conversion and crossing-over, suggesting that it is involved in the initiation of meiotic recombination. The *mei-W68* gene encodes a homolog of Spo11, a finding that provides further support to the notion that Spo11-mediated formation of DSBs is indispensable for the initiation of meiotic recombination among eukaryotes *(66).*

1.5. Current Model for Recombination Induced by DNA Double-Strand Breaks

The DNA DSB repair model for recombination presented by Sun et al. *(105)* provides a conceptual framework for the discussions that follow. In this model (Fig. 1), the ends of the DNA breaks are processed to yield long 3′ single-stranded overhangs, which serve as the substrate for the recruitment of recombination factors. Nucleation of the recombination factors onto the ssDNA tails renders them recombinogenic, leading to a search for an intact DNA homolog, a homologous chromosome or a sister chromatid, and invasion of the DNA homolog to yield heteroduplex DNA. It is important to emphasize that the 3′ ssDNA tails depicted in this model have been detected in numerous studies *(16,102,105),* as have the double Holliday junctions been observed *(95).* In light of this model, we review what is known about the enzymatic reactions that are needed to initiate and complete the recombination process.

2. THE DNA DSB REPAIR REACTION

2.1. DNA End Processing

2.1.1. The Rad50, Mre11, and Xrs2/NBS1 Proteins

RAD50, MRE11, and *XRS2* encoded products are involved in the nucleolytic end-processing reaction that yields the 3′ ssDNA tails (Fig. 1). Results from yeast two-hybrid studies have suggested that these proteins interact with one another *(49).* Rad50 is a member of the *s*tructural *m*aintenance of *c*hromosome (SMC) protein family, possessing coiled coil domains as well as ATP binding motifs. Mre11 exhibits homology to phosphodiesterases, while Xrs2 has a putative forkhead-associated (FHA) domain, which may be involved in protein-protein interactions. Human NBS1, the presumed functional equivalent of yeast Xrs2, possesses an FHA domain and a breast cancer car-

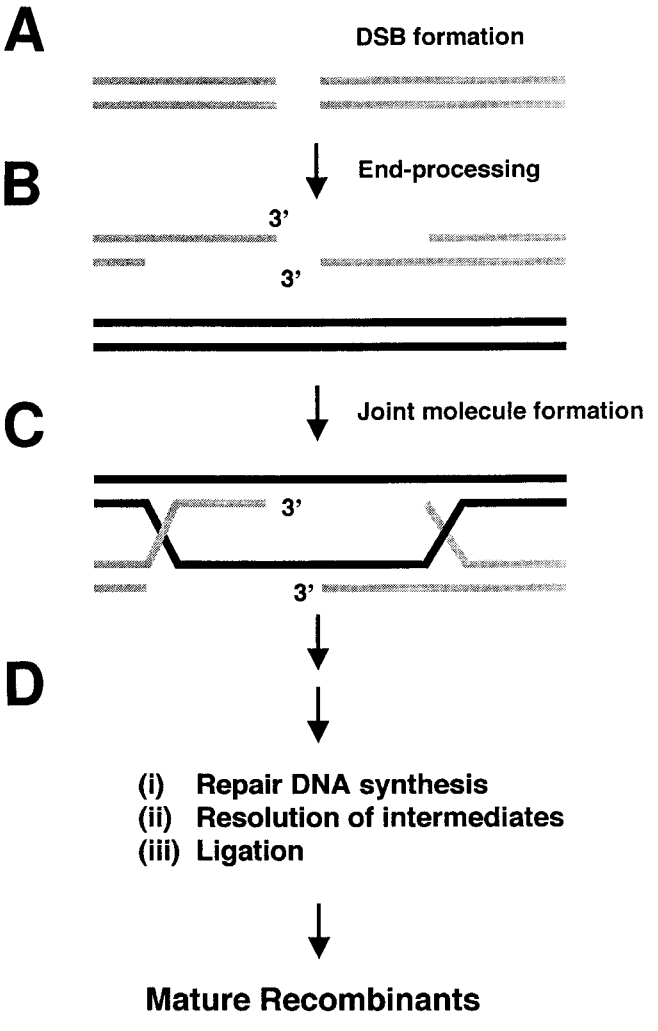


Fig. 1. DSB repair model for recombination. The repair reaction begins with the nucleolytic processing of the break to create a 3′ ssDNA tail for the nucleation of Rad51 and other recombination factors to form a nucleoprotein complex, which has the ability to search for a DNA homolog and mediates DNA strand invasion to form heteroduplex DNA. Repair DNA synthesis serves to replace the genetic information eliminated during end-processing, and resolution of the Holliday junctions followed by DNA ligation then yield mature recombinants.

boxy-terminal (BRCT) domain, which are believed to be involved in protein-protein interactions *(26,32,82).* Human Rad50 and Mre11 have been identified recently *(26,82).* The Rad50 and Mre11 proteins are structurally related to the *Escherichia coli* SbcC and SbcD proteins, respectively *(96).* SbcC and SbcD combine to form a complex that has ATP-independent ssDNA endonuclease and ATP-dependent dsDNA exonuclease activities, as well as an ability to open DNA hairpins *(21).* Consistent with the homology of Rad50 and Mre11 to the SbcC/SbcD nuclease complex, yeast and human Mre11 as well as the protein complex consisting of human Mre11 and associated proteins also possess endonuclease and exonuclease activities (*see* below).

Immunoprecipitation studies indicated that Rad50, Mre11, and three additional protein species having molecular sizes of about 95 kDa, 200 kDa, and 400 kDa are associated in human cell extracts *(26).* Partial purification of the protein complex containing Rad50, Mre11, and associated proteins and peptide microsequencing helped identify the p95 species as the product of the gene mutated in Nijmegen breakage syndrome, a disease characterized by cellular sensitivity to DNA damaging agents, chromosomal fragility, and a high incidence of malignancies. The p95 species is now also referred to as NBS1 or nibrin. The 200 kDa species has been identified as fatty-acid synthase, whose association with the Mre11/Rad50/p95 was judged to be fortuitous *(17),* whereas the identity of the 400 kDa species is currently unknown. It is very likely that NBS1, which shows significant homology to yeast Xrs2 at the amino-terminus, represents the functional analog of Xrs2 in recombination and repair processes *(17,121).*

The biochemical properties of human Mre11 and the protein complex comprising human Rad50, Mre11, and NBS1 are well-characterized. Paull and Gellert *(78)* overexpressed the hMre11 protein in insect cells and purified it to near homogeneity. hMre11 possesses an exonuclease activity that removes mononucleotides from the 3′ termini of dsDNA. Coexpression of hMre11 and hRad50 in insect cells gives a stoichiometric hRad50/hMre11 complex that has four times the exonuclease activity of hMre11 alone. Because hRad50 is not expected to have any nuclease activity, the enhancement of nuclease function seen in the hMre11/hRad50 complex is likely owing to stimulation of hMre11 nuclease activity by hRad50. Whether hRad50 stimulates the hMre11 exonuclease activity by enhancing end recognition or processivity remains to be determined. Like the SbcC/SbcD nuclease complex *(21),* hMre11 also cleaves DNA hairpins *(78).* A more recent study from the Gellert laboratory shows that NBS1 complexes with hMre11 and with hRad50/hMre11. In the presence of ATP, NBS1 modulates the nuclease activities of hMre11 on hairpin substrates, enables hRad50/hMre11 to cleave a 3′ ssDNA overhang, and confers an ability to hRad50/hMre11 for the localized unwinding of duplex DNA *(79).* In addition, it was demonstrated by mutagenesis and biochemical means that hRad50 plays a critical role in the ATP-stimulated endonucleolytic and DNA unwinding activities of the hRad50/hMre11/NBS1 complex. hRPA and the hKu heterodimer, two abundant nuclear DNA binding factors, are found to compete with the hRad50/hMre11/NBS1 complex for DNA substrate sites. Although NBS1 may serve to relay the detection of DNA damage to the cell-cycle checkpoint machinery *(97),* the results of Paull and Gellert *(79)* have demonstrated dramatic effects of NBS1 on the biochemical properties of hRad50/hMre11.

In a parallel study, the hRad50/hMre11/NBS1 complex was purified to near homogeneity from nuclear extracts of Raji cells. However, the 200 kDa species (fatty-acid synthase) and the 400 kDa species did not copurify with the hRad50/hMre11/NBS1 complex *(118).* The purified hRad50/hMre11/NBS1 complex digests covalently closed single-stranded DNA circles and mcks supercoiled DNA. Quantification of the endonuclease activity of the hRad50/hMre11/NBS1 complex on single-stranded vs supercoiled DNA revealed that it is highly specific for single-stranded DNA. It was also shown that the endonucleolytic reaction products have 3′ hydroxyl and 5′ phosphate termini, and that the hRad50/hMre11/NBS1 complex possesses a 3′ to 5′ exonuclease activity *(118).*

Yeast Mre11 is also associated with Rad50 and Xrs2 in a complex *(49,120),* and purified yMre11 also possesses a ssDNA specific endonuclease activity *(34,71,120)* and a 3′ to 5′ exonuclease activity on dsDNA *(34,71,120)* and on ssDNA as well *(120).*

2.1.2. A Model for DNA DSB End-Processing

Extensive genetic studies in *S. cerevisiae* have indicated that the ends of DNA DSBs are processed in an apparent exonucleolytic fashion to yield 3′ ssDNA tails (Fig. 1). One can contemplate at least two possible mechanisms for accomplishing the end-processing reaction. One scenario would involve a classical 5′ to 3′ exonuclease activity that engages the DNA end and then removes nucleotides in reiterative fashion from the DNA strand that contains the 5′ terminus. The other, somewhat more elaborate scheme, would entail the cooperation between an endonucleolytic activity and a helicase function, unwinding the duplex from the extremity to create a ssDNA region acted on by the endonucleolytic function. The two possible mechanisms of end-processing may not be mutually exclusive, as a nuclease/helicase complex may possess all the activities required for mediating the end-processing reaction via both routes, or alternatively, an exonuclease and an endonuclease/helicase complex may independently provide end-processing functions in parallel reactions.

Given that the Mre11-associated protein complex is apparently devoid of a significant 5′ to 3′ exonuclease activity *(34,71,78,118),* the most pertinent query then becomes: How may the Mre11 nuclease activities be utilized for the end-processing reaction? One plausible scenario is that the 3′ to 5′ exonuclease activity, at least under some circumstances, makes a short 5′ single-stranded overhang for the loading of a DNA helicase, or one of a number of alternate helicases, to initiate DNA unwinding for creating a branched DNA structure for the Mre11 endonuclease function to act, as depicted in Fig. 2. We favor this hypothetical model of DSB end-processing for two reasons. First, it reconciles the polarity of DNA end-processing observed in vivo with the known biochemical properties of purified Mre11 and the Mre11/Rad50/NBS1 complex. Second, this model shares major features with the known mechanism of the DNA end-processing complex RecBCD during recombination processes in *E. coli,* which creates a recombinogenic 3′ ssDNA tail via DNA unwinding and internal scission of the unwound, 5′ overhanging DNA strand (reviewed in *57).* The validation of this working model (Fig. 2) will require the identification of the putative DNA helicase(s) that physically and functionally interacts with the Mre11 protein complex.

During the processing of meiotic DSBs, it is believed that the Mre11 endonuclease activity removes Spo11 that remains covalently attached to the 5′ termini of the DNA ends. In this case, the result of Spo11 removal may be the generation of a short 3′ overhang to be utilized for the loading of a DNA helicase to initiate DNA strand separation *(71,120).*

2.1.3. Multifunctional Nature of the Mre11-Associated Protein Complex

As mentioned earlier, in addition to mediating the DNA end processing reaction, the Rad50/Mre11/Xrs2 complex, in conjunction with Spo11 and other factors, is also required for the formation of DNA DSBs at the initiation sites of meiotic recombination (*see 54* for a discussion). Subsequent to the formation of meiotic DSBs, it is believed that Spo11 remains covalently attached to the 5′ termini of the DNA ends, and that the removal of the covalently conjugated Spo11 is mediated by the Mre11 endonucleolytic activity *(77).* Genetic studies *(13,70,93)* have also indicated a role for Rad50, Mre11, and Xrs2 in the DNA end-joining pathway of DSB repair as well. Remarkably, these three proteins have also been linked to the maintenance of telomere length *(13,77).* In mediating biological functions distinct from DNA DSB end-processing, Mre11 and

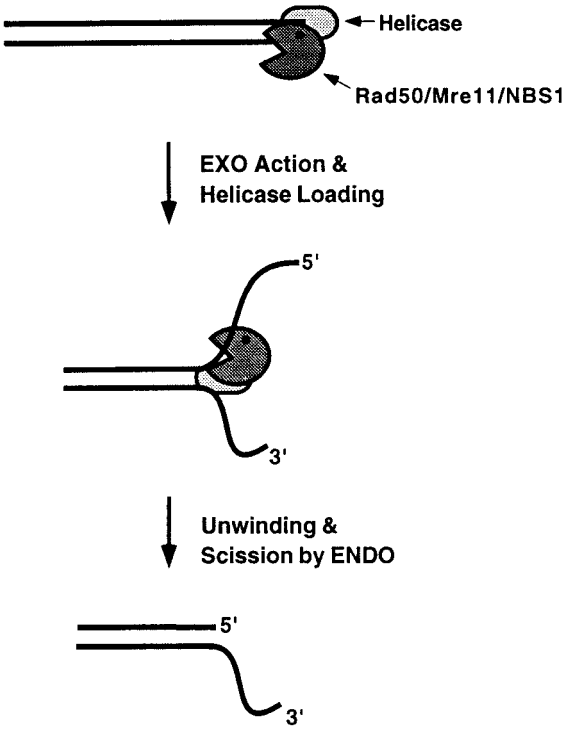


Fig. 2. A hypothetical model for DSB end-processing. The first step of DNA end-processing is postulated to be the creation of a short 5′ ssDNA overhang by the 3′ to 5′ exonuclease activity of the Rad50/Mre11/NBS1 complex. This is followed by the loading of a DNA helicase, DNA strand separation by the helicase, and endonucleolytic cleavage of the 5′ overhanging DNA strand to generate the 3′ ssDNA tail. This model may not apply in the case of the processing of DSBs generated during meiosis (*see* text for details).

associated proteins may provide a molecular scaffold for the recruitment of additional protein factors and the assembly of the appropriate protein complexes. This suggestion is supported by the observation that some the DNA end-joining events affected in *rad50, mre11,* and *xrs2* mutants do not actually require nucleolytic end-processing *(13).* In fact, inactivation of the Mre11 nuclease function has no discernible effect on end-joining and telomere-length maintenance *(71).* An intriguing genetic interaction between the Mre11/Rad50/Xrs2 complex and the Srs2 DNA helicase has been observed by Klein and colleagues; haploid *srs2 rad50, srs2 xrs2,* and *srs2 mre11* mutants have a severe growth deficiency and the mutant diploids are inviable. The precise reason for the synthetic growth deficiency and inviability remains to be elucidated, but it appears to be unrelated to the telomere maintenance function of Rad50/Mre11/Xrs2 *(56).*

3. HETERODUPLEX DNA FORMATION

Genetic exchange involves the formation of a heteroduplex DNA intermediate between the recombining homologous chromosomes (Fig. 1; *see* 112 for a discussion). Substantial genetic and biochemical evidence has implicated the *RAD51, RAD52, RAD54, RAD55, RAD57,* and *TID1/RDH54* genes in heteroduplex DNA formation;

Table 1
Factors that Function in Heteroduplex DNA Formation

Yeast factor	Known role	Human equivalent	Prokaryotic equivalent
Rad51	Recombinase	hRad51	RecA in *E. coli* UvsX in T4
Rad52	Mediator	hRad52	RecO/RecR in *E. coli* UvsY in T4
Rad55/Rad57	Mediator	XRCC2, XRCC3, Rad51B, Rad51C, and Rad51D[a]	RecO/RecR and UvsY
Rad54	Synaptic factor	hRad54	Not known
Rdh54	Synaptic factor[a]	Not yet identified	Not known
Rad59	Currently unknown	Not yet identified	Not known
Dmc1	Recombinase[b]	hDmc1	RecA and UvsX

[a] These proteins exhibit homology to the hRad51. Based on the paradigm established with yeast Rad55/Rad57 complex *(109),* we suspect that some or all of these human factors may also function as mediators with hRad51. As for yeast Rdh54, there is currently a paucity of information concerning its biochemical function. We tentatively assign a synaptic function to Rdh54, based on its similarity to Rad54 and on genetic observations that the two appear to play somewhat overlapping roles in recombination and repair.

[b] Based on studies with the human Dmc1 protein *(59).*

whether *RAD59* also has a role remains to be established. The basic mechanism for heteroduplex DNA formation is very likely conserved among eukaryotes, as structural/functional homologs of the yeast Rad51, Rad52, and Rad54 proteins have been identified in humans (*see 51* for a discussion), and characterization of the human proteins *(7,8,39,89,111)* revealed functional properties similar to those of their yeast counterparts *(73,74,83,100,101,103,104,106,107,110).* The recombination factors that function in heteroduplex DNA formation are listed in Table 1.

3.1. Role of Various Recombination Factors in Heteroduplex DNA Formation

The enzymatic process responsible for creating heteroduplex DNA during recombination is referred to as homologous DNA pairing and strand exchange. The family of proteins that mediate homologous DNA pairing and strand exchange, including *E. coli* RecA, bacteriophage T4 UvsX, and Rad51 from yeast and humans, are called "recombinases." Based on biochemical studies of prokaryotic and eukaryotic recombinases, some salient features concerning the activities of these enzymes and the homologous pairing and strand exchange reaction have emerged, as highlighted later.

3.1.1. The Rad51 Recombinase

The *RAD51* encoded product from both yeast and humans possesses homologous DNA pairing and strand-exchange activities *(7,39,103,106).* Biochemical studies have strongly suggested that, like the prokaryotic recombinases, the Rad51-mediated homologous DNA pairing and strand exchange reaction proceeds via two distinct stages, termed presynaptic and synaptic phases (reviewed in *57).*

3.1.2. The Presynaptic Phase

In the presynaptic phase, Rad51 polymerizes on ssDNA to form a right-handed protein filament that has a highly regular pitch (about 95 angstroms) and in which the DNA

is held in an extended conformation (axial rise of about 5 angstroms per base or base pair as compared to 3.4 angstroms per base pair for B form duplex DNA). The Rad51-DNA nucleoprotein filament is almost identical in overall dimensions and appearance to the equivalent filament of RecA protein *(75,107).* Biochemical studies have indicated that formation of heteroduplex DNA with the incoming duplex DNA partner occurs within the confines of this nucleoprotein filament *(107).* Although Rad51 possesses a DNA-dependent ATPase activity, the formation of the presynaptic nucleoprotein filament requires only ATP binding *(108),* as for RecA *(57).* The assembly of the Rad51 presynaptic filament is stimulated by the heterotrimeric ssDNA binding factor RPA, when RPA is added to the in vitro reactions after Rad51 has been allowed to nucleate onto the ssDNA *(103,109).* Paradoxically, when added to the ssDNA before or together with Rad51, RPA interferes with the assembly of the presynaptic filament, leading to an inhibition of heteroduplex DNA formation *(109).* Specific protein factors, termed mediators *(51,110),* have been identified that function to facilitate the assembly of the Rad51 filament when RPA is competing for DNA binding sites with Rad51. These mediators, Rad52 protein and the Rad55/Rad57 complex (*see* below), are functionally equivalent to *E. coli* RecO/RecR complex *(119)* and T4 UvsY *(40,46).*

3.1.3. The Synaptic Phase

In this reaction phase, the Rad51-ssDNA nucleoprotein filament conducts a search for DNA homology, resulting in the synapsis or pairing with the homolog, and formation of heteroduplex DNA with the homolog. Concerning the mechanism of the DNA homology search and pairing process, some clues have been gleaned from studies conducted with the RecA-ssDNA nucleoprotein filament. In this case, the nucleoprotein filament contains a second DNA binding site that accommodates the incoming duplex DNA molecule. Following the incorporation of the duplex molecule into the RecA-ssDNA nucleoprotein filament through nonhomologous contacts, homology is found, and alignment of the two recombining molecules is established through a series of transient, short heteroduplex joints called "paranemic" joints. When a free end is present either in the ssDNA or the dsDNA molecule, intertwining of the ssDNA strand with the complementary strand in the duplex partner occurs, resulting in the formation of a stable joint molecule called a "plectonemic" joint. Subsequent to the formation of the nascent plectonemic joint molecule, unidirectional branch migration, or DNA strand exchange, leads to the extension of the heteroduplex joint *(2,14,23,57,88).* It is likely that this paradigm (Fig. 3) will be applicable to Rad51 as well. In yeast, the rate of homologous DNA pairing by Rad51 is greatly accelerated by Rad54 (*see* Subheading 3.3.1.).

3.2. Mediators

As described earlier, RPA is required in the synaptic phase of the homologous pairing and strand exchange reaction. RPA is believed to remove the secondary structure in ssDNA that would otherwise impede the Rad51 filament assembly process *(103,107).* The stimulatory effect of RPA is seen most clearly when it is added to the reaction after Rad51 has already nucleated onto ssDNA. If RPA is added before or with Rad51, a pronounced inhibition of pairing and strand exchange ensues *(109).* This and other observations *(103,109)* have led to the deduction that RPA, in addition to its stimulatory role, can also compete with Rad51 for binding sites on the ssDNA substrate *(103,109).* The

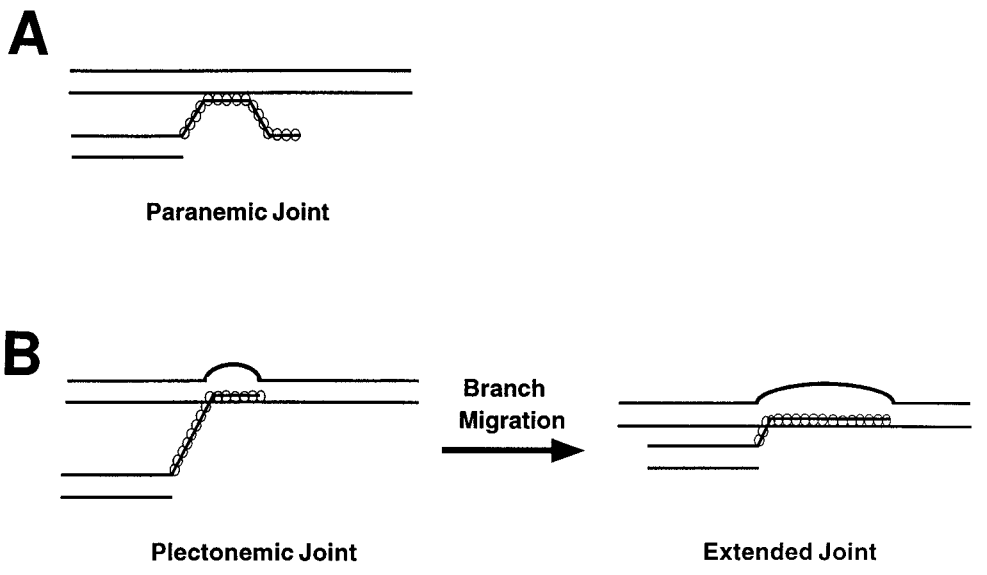


Fig. 3. DNA joints in the formation of heteroduplex DNA. **(A)** The first homologous joints formed between the recombining DNA molecules are paranemic in nature. The paranemic joints (only one such joint is shown) are unstable but are thought to be important intermediates that lead to the capture of the incoming homolog and the homologous alignment of the recombining DNA molecules. **(B)** Subsequent to homologous alignment of the DNA molecules, the free DNA end is located and the formation of a stable plectonemic linkage becomes possible. Branch migration results in extension of the plectonemic joint and the heteroduplex DNA region. The circles in (A) and (B) denote Rad51 molecules. It should be emphasized that the nucleoprotein complex that conducts the DNA homology search and pairing steps very likely contains other protein factors of the *RAD52* group, including Rad52, Rad54, and Rad55-Rad57 in yeast (*see* text for details).

depression of homologous DNA pairing and strand exchange by RPA suggests that specific ancillary factors must function with Rad51 in vivo to overcome the competition posed by RPA. Indeed, the Rad52 protein and the Rad55/Rad57 complex have been shown to facilitate the assembly of the Rad51-ssDNA nucleoprotein filament when RPA is competing for DNA binding sites with Rad51 *(74,100,109,110).* Consistent with the biochemical results, formation of meiotic Rad51 nuclear foci, believed to be sites of ongoing recombination, is strongly dependent on the *RAD52, RAD55,* and *RAD57* genes *(36).* The relevant information concerning these mediators is described in Subheading 3.2.1.–3.2.2.

3.2.1. Rad52 Protein

Rad52 is a ssDNA binding protein *(73,101)* and it forms a complex with Rad51 *(69,98,110).* Addition of Rad52 to a homologous DNA pairing and strand-exchange reaction overcomes the inhibition posed by RPA *(74,100,110),* indicating a mediator function in the protein. Rad52 is of much lower cellular abundance than Rad51, and interestingly, an amount of Rad52 approx one tenth that of Rad51 is optimal for its mediator function *(110).* In the presence of Rad52, RPA is still required for the optimal pairing and strand exchange, thereby excluding the possibility that Rad52 is simply replacing RPA in vitro

(110). Taken together, the results suggest that a complex of Rad51/Rad52 bound to ssDNA provides a priming effect for the recruitment of free Rad51 molecules. Genetic studies have indicated that Rad52 also functions in a specialized pathway of homologous recombination termed single-strand annealing or SSA, which involves the annealing of homologous DNA single strands (*45,77,* and *see* Subheading 6.). Consistent with the genetic results, Rad52 mediates the annealing of DNA strands *(73),* in a reaction that is stimulated by RPA *(101,104).* Recent studies have shown that human Rad52 binds dsDNA breaks and promotes end-to-end DNA interactions in vitro *(29).* A model was proposed that Rad52 binds to DSBs in vivo and mediates repair either by SSA mechanism or by Rad51-mediated pathway by recruiting Rad51 to the breaks *(29).* The strand-annealing activity of Rad52 could also be utilized for the formation of a short heteroduplex joint for priming DNA synthesis, which may be important for a mechanism of DNA repair termed DNA break-induced replication or BIR *(65).*

3.2.2. Rad55/Rad57 Complex

Both Rad55 and Rad57 share some limited homology to Rad51, especially within the sequence motifs involved in the binding and hydrolysis of nucleoside triphosphates *(61).* In agreement with two-hybrid results, which suggested an interaction between Rad55 and Rad57 *(41,48),* stoichiometric amounts of these proteins co-immunoprecipitate from yeast cell lysate, indicating that the proteins are stably associated in a complex *(109).* Purification of the Rad55/Rad57 complex has revealed that it is heterodimeric *(109).* The Rad55/Rad57 heterodimer is also capable of overcoming the inhibition by RPA, indicative of a mediator function of the heterodimer in the assembly of the presynaptic Rad51-ssDNA nucleoprotein filament. The Rad55/Rad57 heterodimer is of much lower cellular abundance than Rad51, and amounts of purified Rad55/Rad57 substoichiometric to that of Rad51 are sufficient for mediator function. In the presence of the Rad55/Rad57 heterodimer, RPA is still needed for efficient pairing and strand exchange *(109).* Whether Rad55/Rad57 acts by a mechanism similar to that of Rad52 or by a distinct mechanism remains to be determined. *rad55* and *rad57* single mutants are as defective in recombination and repair as the double mutant, which emphasizes that Rad55 and Rad57 function together in the same step during recombination, and also nicely underscores the finding that the two proteins are associated as a heterodimer. A notable feature about the *rad55* and *rad57* mutants is that they are cold sensitive for recombination *(62).*

3.3. Factors That Function in the Synaptic Phase

3.3.1. Rad54 Protein

Rad54 belongs to the Swi2/Snf2 protein family, members of which are involved in diverse chromosomal processes including transcription and repair *(30,80,94).* Rad54 is of much lower cellular abundance than Rad51 *(47).* Consistent with the presence of Walker-type nucleotide binding motifs in Rad54 *(31),* purified Rad54 has a robust ATPase activity that is completely dependent on DNA, dsDNA in particular, for its activation. However, Rad54 does not appear to possess a classical DNA helicase activity *(83).* Rad54 physically interacts with Rad51 *(20,47,83),* and the addition of Rad54 protein to a homologous DNA pairing reaction results in a dramatic stimulation of the pairing rate *(83).* Whereas Rad51 is incapable of mediating pairing between a linear ssDNA

molecule and a covalently closed duplex to form a D-loop, the inclusion of Rad54 protein renders D-loop formation highly efficient. Control experiments have shown that Rad54 by itself is devoid of homologous DNA pairing activity *(83).* Human Rad54 also interacts with hRad51 *(38),* possesses a dsDNA-dependent ATPase activity *(111),* and its expression in a yeast *rad54* mutant partially complements the MMS sensitivity of the mutant *(52).* Mouse Rad54 forms nuclear foci that co-localize with mouse Rad51 upon treatment of cells with ionizing radiation, and the formation of the DNA damage-induced Rad51 nuclear foci is dependent on Rad54 *(114).* Human Rad54 induces a conformational change in the duplex DNA in an ATP-hydrolysis-dependent manner, as revealed in a topological unwinding assay *(114).* Yeast Rad54 has also been found to induce a similar conformational change in duplex DNA upon hydrolyzing ATP *(84).* The ability to alter the conformation of the DNA double home is likely to be important for the promotion of the homologous DNA pairing reaction *(84).*

3.1.2. Rdh54 Protein

The yeast Rdh54/Tid1 shows 35% identity to Rad54. Although the *rdh54*Δ mutation confers only slight sensitivity to MMS, it increases the MMS sensitivity of a *rad54*Δ strain. Likewise, the *rad54*Δ *rdh54*Δ double mutant is more impaired in meiosis than either single mutant alone *(55,99).* In a separate study, Dresser et al. *(28)* identified Rdh54 in a two-hybrid screen as a protein that interacts with the meiosis-specific Rad51 homolog Dmc1. These observations suggest that Rdh54 functions with Rad51 in gene conversion between homologs in mitotic cells *(55)* and with both Dmc1 and Rad51 in interhomolog recombination in meiotic cells *(55,99).* Like Rad54, Rdh54 has Walker-type nucleotide binding motifs. Considering the involvement of Rdh54 in recombination and repair as well as its structural similarity to Rad54, one suspects that Rdh54 also affects heteroduplex DNA formation during the synaptic phase.

3.2. Other Recombination Factors

3.2.1. Rad59 Protein

Rad59 functions in intrachromosomal recombination and DNA DSB repair, apparently in a pathway distinct from that dependent on Rad51. Rad59 shows some homology to Rad52, and overexpression of *RAD52* partially suppresses the recombination deficiency and γ-ray sensitivity of a *rad59* mutant *(4).* The biochemical function of Rad59 protein in recombination and repair remains to be determined.

3.2.2. Dmc1, a Meiosis Specific Recombinase

Yeast Dmc1 is highly homologous to Rad51. *DMC1* is required for meiotic recombination and chromosomal disjunction, and its expression is restricted to meiosis. Consistent with this expression pattern, deletion of *DMC1* produces no discernible mitotic phenotype *(11).* Dmc1 colocalizes with Rad51 in nuclear foci during meiosis *(10).* Dmc1 structure and function appear to be highly conserved among eukaryotic organisms *(85,124).* Human Dmc1 has been purified and shown to possess homologous DNA pairing activity *(59).* The fact that amounts of hDmc1 stoichiometric to that of ssDNA are required for the optimal rate of homologous pairing argues for the possibility that hDmc1 promotes pairing via the formation of a stoichiometric protein complex on ssDNA, likely a nucleoprotein filament *(59).* The level of homologous pairing that can be achieved by hDmc1 protein is relatively low, suggesting that ancillary factors may

function with hDmc1 to attain optimal activity. Whether Dmc1 from yeast and human cooperates with the same set of recombination factors that function to enhance the recombinase activity of Rad51 remains to be determined.

3.3 Human Proteins Homologous to Rad51

The chinese hamster ovary (CHO) cell lines irs1 and irs1SF, defective in the *XRCC2* and *XRCC3* genes, respectively, are sensitive to ionizing radiation and to DNA crosslinking agents *(50,115,117).* Analyses of the cloned *XRCC2* and *XRCC3* cDNAs have revealed that their products are homologous to Rad51 *(18,60).* In addition, XRCC3 has been shown to interact with Rad51 in the yeast two-hybrid system and to co-immunoprecipitate with Rad51 from cell extract *(60).* Interestingly, XRCC3 has been shown in the Bishop laboratory to be required for the formation of DNA damage-induced Rad51 nuclear foci *(12).* Taken together, it seems plausible to suggest that XRCC3 functions with Rad51 in recombination and DNA repair processes. Given the phenotypic similarity of the irs1 cell line to the irs1SF cell line and the homology of XRCC2 to XRCC3 and Rad51, it is likely that XRCC2 is also a component of the recombination protein machine that contains the latter two proteins. Three additional Rad51-homologous proteins, Rec2/Rad51B *(3,90),* Rad51C *(27),* and Rad51D *(86),* have been identified based on their amino acid sequence homology to Rad51. Rad51C has been found in the yeast two-hybrid system to interact with XRCC3 and Rad51B *(27).* In *S. cerevisiae,* the Rad51-homologous proteins Rad55 and Rad57 combine to form a heterodimer (*see* Subheading 3.2.2.) that functions to promote the assembly of the Rad51-ssDNA nucleprotein filament *(109).* There is a distinct possibility Rad51B, Rad51C, Rad51D, XRCC2, and XRCC3 also form complexes with one another and function to enhance hRad51-ssDNA nucleoprotein filament assembly.

4. REPAIR DNA SYNTHESIS, RESOLUTION OF RECOMBINATION INTERMEDIATES, AND DNA LIGATION

As can be seen in the model in Fig. 1, concomitant with heteroduplex DNA formation, repair DNA synthesis replaces the genetic information eliminated during the end-processing reaction. Holmes and Haber *(43)* have presented evidence that during conversion of the mating-type information at *MAT,* the repair DNA synthesis step requires the concerted action of DNA polymerases α, δ, and ε, leading to the suggestion that the repair synthesis reaction entails the establishment of both leading and lagging DNA strands. Logic would predicate that the repair DNA synthesis reaction be coupled to the strand-invasion reaction that yields heteroduplex DNA. In other words, it is very likely that the DNA polymerases and accessory factors are actively recruited to the sites of recombination. At present, little is known about how the DNA synthesis proteins are recruited to recombination sites, and if any of the known recombination factors serves to recruit them.

Two genes, *MSH4* and *MSH5,* appear to specifically affect crossover or reciprocal recombination and may therefore encode factors that influence processing of a DNA intermediate, such as the Holliday junction, critical for the generation of crossover recombinants. Expression of *MSH4* and *MSH5* is seen only in meiosis, and in *S. cerevisiae* strains mutated for these two genes, the levels of meiotic-gene conversion and postmeiotic segregation appear to be normal at the majority of the loci examined, but crossover recombination is reduced two- to threefold. As a result of the deficiency in

crossover recombination, *msh4* and *msh5* mutants are partially defective in chromosome disjunction during meiosis I, giving rise to a sporulation deficit and low spore viability. The *MSH4* and *MSH5* genes show epistasis in meiotic crossover recombination, suggesting that their encoded products function in the same biological pathway or reaction *(42,92).* More recent results have indicated that Msh4 and Msh5 exist as a complex in yeast cells *(87)* and have implicated a role for the mismatch repair factor Mlh1 in the Msh4/Msh5-dependent pathway of crossover recombination *(44).* Aside from a possible function of the Msh4-Msh5 protein complex in Holliday-junction processing, relatively little is known about other nuclear recombination factors that promote branch migration and resolution of the Holliday junction and other recombination intermediates.

Following the resolution of recombination intermediates, DNA ligation complete the recombination process by sealing the DNA nicks. Of the two DNA ligases in yeast, ligase I and ligase IV, existing evidence suggests that ligase I, the product of the *CDC9* gene, is involved in recombinational DNA repair *(33).*

5. MODULATION OF DSB REPAIR BY TUMOR SUPPRESSORS

There is emerging evidence that the recombination machinery in mammalian cells is subject to modulation by genes that are critical for cancer suppression. In addition to the *NBS1* gene, which encodes an integral component of the Mre11-associated nuclease complex, recent studies have implicated the gene mutated in ataxia-telangiectasia *(ATM)* and the breast tumor-suppressor gene *BRCA2* in recombination and repair.

Ataxia-telangiectasia (A-T) is an autosomal recessive disease characterized by neurodegeneration, cancer predisposition, immunodeficiency, gonadal atrophy, and hypersensitivity to ionizing radiation (reviewed in *58* and *68). ATM* encodes a member of the PI3-like kinase family that includes proteins involved in cell-cycle checkpoint control, meiosis, and V(D)J recombination (reviewed in *125*). ATM protein phosphorylates p53, and this modification is believed to be important for the p53-dependent DNA damage induced G1/S checkpoint *(5,15).* However, because p53 is not essential for other ATM-mediated checkpoints, it is likely that other checkpoint factors are also modified in an ATM-dependent manner. In addition to its checkpoint function, ATM may be directly involved in DNA repair processes, as A-T cells remain hypersensitive to ionizing radiation under conditions where the checkpoint function is dispensable *(116).* Furthermore, most A-T patients and all ATM-deficient mice of both sexes are infertile owing to the absence of mature gametes (reviewed in *58).* In mice, ATM is required for meiosis as early as the leptonema stage of meiosis I. In the absence of ATM, Dmc1 and Rad51 mislocalize to chromatin instead of the developing synaptonemal complex *(6).* The mislocalization of Rad51 appears to reflect a requirement for the ATM-dependent kinase cascade in the phosphorylation of Rad51 protein (EL, unpublished observation). A high frequency of spontaneous recombination has been observed in A-T cells *(63,67),* and in addition, DNA end-joining also appears to be aberrant in these cells *(24).* It is interesting to note that A-T and NBS cells share many cellular phenotypes, thus NBS was long-regarded as an A-T variant (reviewed in *97).*

Mutations in *BRCA2* account for a large proportion of familial breast cancers (reviewed in *126*). Cultured cells become sensitive to γ-irradiation upon downregulation of BRCA2 protein expression by treatment with BRCA2 antisense oligonucleotides *(1).* In addition, fibroblasts established from mutant *Brca2* mouse embryos are specifically

sensitive to γ-irradiation *(22,72,76).* BRCA2 protein associates with Rad51, and the interaction domain has been mapped to the BRC repeats in BRCA2 *(19,53,122).* The human pancreatic adenocarcinoma Capan-1 cells, which lack one copy of the *BRCA2* gene and contain a truncating mutation (6174delT) in the other *BRCA2* allele *(37),* are hypersensitive to various DNA damaging agents. The MMS sensitivity of Capan-1 cells is complemented by the introduction of *BRCA2* cDNA, but not by a truncated cDNA with the BRC repeats deleted. These observations have provided evidence that the interaction between Rad51 and the BRC repeats in BRCA2 is important for DNA repair *(19).*

6. HOMOLOGOUS RECOMBINATION BY SINGLE-STRAND DNA ANNEALING

When homologous genetic elements are arranged as direct repeats on the same chromosome or DNA molecule, a plasmid for instance, a DSB introduced between the DNA repeats is processed to yield 3′ overhanging single-stranded tails that are partially complementary to each other, and thus have the potential of undergoing annealing to yield heteroduplex DNA. Once hybridized, the remaining nonhomologous, overhanging single-stranded tails are trimmed nucleolytically. Gap filling by a DNA polymerase followed by DNA ligation would yield a recombinant DNA molecule that has some of the original DNA sequence deleted. This type of homologous recombination is termed single-strand annealing or SSA *(45,77).* Interestingly, the SSA pathway of recombination shows a dependence on *RAD52* but not on the other members of the *RAD52* epistasis group, with the possible exception of *RAD59.*

7. EPILOGUE

Now that many of the main players that stage the production of DNA DSB, DNA end-processing, and heteroduplex DNA formation have been identified in *S. cerevisiae,* it is expected that rapid progress will be made on understanding these individual enzymatic steps at the mechanistic level. Major challenges lie with identifying the protein components involved in the later stages of recombination processes. Attempting to elucidate the mechanism of action of various tumor suppressors in modulating the efficiency of the DNA DSB repair machinery will be a highly interesting area of research in the coming years.

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7

The MRE11-RAD50 Complex

Diverse Functions in the Cellular DNA Damage Response

John H. J. Petrini, Richard S. Maser, and Debra A. Bressan

1. INTRODUCTION

In recent years, progress toward understanding the mechanisms and molecules with which mammalian cells respond to DNA double-strand breaks (DSBs) has been dramatic. This is attributable in part to the analysis of DSB repair-deficient rodent cell lines, which led to the isolation and characterization of the DNA-PK complex, XRCC4, DNA ligase IV, and others *(3,43,49,59,60,79,83,100,103,112).* The gene products thus identified have provided many important insights regarding the functions that maintain genomic integrity in the face of genotoxic stress. These studies have also shed light on DNA recombination pathways that diversify genetic information in the establishment of the immune repertoire. Nonetheless, the bulk of our current understanding of DNA recombination pathways has come from genetic and biochemical studies in *Saccharomyces cerevisiae* and bacterial systems. As an alternative to phenotype-driven analysis, a number of investigators have pursued the identification of mammalian counterparts to bacterial and *S. cerevisiae* recombinational DNA repair proteins to examine the cellular DNA damage response in mammals *(46,80).* The potential of this comparative approach has been most impressively realized in the functional analysis of the yeast and mammalian Rad51 proteins, which are homologs of the bacterial RecA protein *(9,38,96).* More recently, the MRE11-RAD50 protein complex, with homologs in bacteria, *S. cerevisiae,* and mammals, has emerged as a central player in the DNA transactions that preserve genomic integrity in yeast and mammalian cells.

This chapter includes descriptions of *S. cerevisiae,* mouse, and human genes and their protein products. For the sake of consistency, the following nomenclature will be used throughout:

Wild-type gene or locus (human, mouse or yeast)……*hRAD50, mRad50,* or *ScRAD50*
Mutant gene or locus (human, mouse or yeast)……*hrad50, mrad50,* or *Scrad50*
Protein product (human, mouse or yeast)……hRAD50, mRAD50, or ScRad50p

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2. OVERVIEW OF THE *S. CEREVISIAE* AND MAMMALIAN MRE11-RAD50 PROTEIN COMPLEXES

The existence of the Mrellp-Rad50p protein complex was first suggested by the striking similarity of the *Scmrell, Scrad50,* and *xrs2* phenotypes *(1,2,40,42,80,84).* Subsequently, physical association of the *S. cerevisiae* proteins and of the human hMRE11 and hRAD50 gene products was established *(26,109). hMRE11* was first identified in a two-hybrid screen for proteins that interact with DNA ligase I, although the physiological relevance of this interaction is uncertain *(81).* The *hRAD50* cDNA is part of a collection of cDNAs originating from the chromosomal region spanning 5q23 to 5q31 that was isolated in an effort to identify a tumor-suppressor gene involved in myelodysplasia and myeloid leukemia that maps to that region. The *hRAD50* gene was identified among these cDNAs on the basis of its similarity to the *S. cerevisiae* gene as well as to a *Caenorhabditis elegans* EST *(26).* The *hRAD50* locus, which maps to 5q31, was subsequently ruled out as the locus of the AML tumor suppressor *(53).*

Phenotypic analyses implicate the *S. cerevisiae* Mre11p-Rad50p-Xrs2p complex in diverse aspects of both meiotic and mitotic recombination pathways. In meiosis, the complex is important prior to the formation of the initiating DSBs, apparently playing a role in modulating chromatin structure at DSB initiation sites *(75)* as well as in recruiting the protein(s) that mediate the actual cleavage event *(47).* Meiotic DSBs are formed by Spo11p, which becomes covalently attached via a phosphoester linkage to the 5′ ends at DSBs *(6,47).* This covalent association is normally transient, but is stabilized in certain *Scmre11* and *Scrad50* backgrounds (the *mre11S* and *rad50S* alleles) *(2,70,106).* This indicates that the complex is also important following DSB formation, and suggests that it mediates the endonucleolytic cleavage of Spo11p from the ends of initiating DSBs. The functions, if any, of the hMRE11-hRAD50 complex in mammalian meiosis remain to be established.

In mitotic cells, the ScMre11p-ScRad50p-Xrs2p complex functions in nonhomologous endjoining (NHEJ) *(35,46,80).* At first glance, homologous recombination seems to be normal in mutants of the complex; in fact *Scmre11, Scrad50,* and *xrs2* mutants exhibit increased rates of spontaneous heteroallelic recombination. However, the complex may play a global role in stabilizing or potentiating chromatid interactions during recombinational DNA repair, and thus function in homologous recombination as well as DNA endjoining *(14,68).* It is conceivable that this function is also relevant to the increased rate of chromosome loss observed in the corresponding mutants *(10,16,40,68).*

In mammalian cells, insights regarding the functions of the MRE11-RAD50 complex have come from genetic, biochemical, and cytological analyses. The mammalian complex consists of at least four members (Fig. 1). Whereas Mre11p and Rad50p are highly conserved, Xrs2p has been replaced in the mammalian complex by p95 (also known as NBS1 or Nibrin), with which it shares only limited similarity *(20,26).* Unlike MRE11 and RAD50, p95 is not essential for cellular survival *(61,116).* p95 deficiency forms the molecular basis of the rare chromosomal instability syndrome, Nijmegen breakage syndrome (NBS), also known as the ataxia telangiectasia (A-T) variant syndrome *(20,95).* The cellular phenotypic features of NBS suggest that p95 deficiency compromises the ability of cells to detect and signal the presence of DNA damage, thus implicating the hMRE11-hRAD50 complex in this function. Consistent with this role for the human complex, cytological analyses have unambiguously demonstrated that

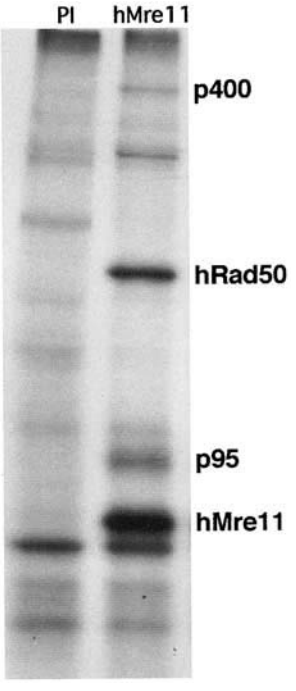


Fig. 1. The hMre11-hRad50-p95 complex. Human lymphoblast cells were metabolically labeled with ^{35}S-methionine, then immunoprecipitated with either preimmune serum (PI) or with hMre11 antiserum (hMre11). The immunoprecipitates were resolved by sodium dodecyl sulfate (SDS) gel electrophoresis, revealing the members of the hMre11-hRad50-p95 complex. Equivalent results are obtained using antisera derived against any of the known complex members. The identity of p400 remains unknown. A similar complex, comprised of at least ScMre11, ScRad50, and Xrs2p, exists in *S. cerevisiae (109).*

the complex associates with ionizing radiation (IR)-induced DNA DSBs early in the cellular DNA damage response *(64,71).*

3. IN VITRO ACTIVITIES

The Mre11p and Rad50p homolog exhibit impressive similarity to SbcD and SbcC, respectively, components of the bacterial exonuclease SbcCD *(94).* SbcCD is a large multimeric complex that exhibits ATP-dependent exonuclease activity, as well as ATP-independent endonuclease activity, both of which require Mn^{2+} as a cofactor *(22).* SbcD is the catalytic subunit of SbcCD, and exhibits strong similarity to the Mre11p homolog (Fig. 2). The sequence similarity among this phylogenetically diverse group of proteins primarily comprises four discrete domains, three of which are also found in protein phosphatases such as λ phosphatase *(55).* Structural studies of λ as well as the mammalian phosphatases demonstrate that residues within each of the three conserved domains are involved in metal-ion binding, which is in turn critical for enzymatic activity *(30,119,120).* The fourth domain is unique to the nucleases *(55).*

A number of laboratories have shown that both the yeast and human MRE11 proteins exhibit nuclease activity in vitro *(27,69,77,105,109).* The activities observed include 3′–5′ exonuclease activity on double-stranded DNA (dsDNA) substrates as well as

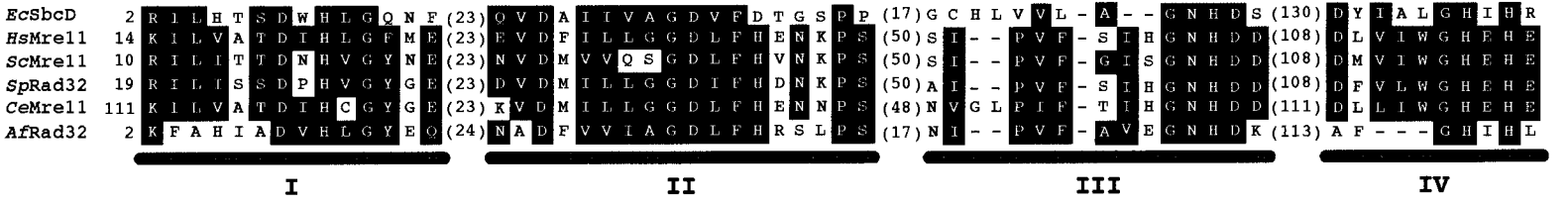

EcSbcD 2 RILHTSDWHLGQNF (23) QVDAIIVAGDVFDTGSPP (17) GCHLVVL-A--GNHDS (130) DYIALGHIHR
HsMre11 14 KILVATDIHLGFME (23) EVDFILLGGDLFHENKPS (50) SI--PVF-SIHGNHDD (108) DLVIWGHEHE
ScMre11 10 RILITTDNHVGYNE (23) NVDMVVQSGDLFHVNKPS (50) SI--PVF-GISGNHDD (108) DMVIWGHEHE
SpRad32 19 RILISSDPHVGYGE (23) DVDMILLGGDIFHDNKPS (50) AI--PVF-SIHGNHDD (108) DFVLWGHEHE
CeMre11 111 KILVATDIHCGYGE (23) KVDMILLGGDLFHENNPS (48) NVGLPIF-TIHGNHDD (111) DLLIWGHEHE
AfRad32 2 KFAHIADVHLGYEQ (24) NADFVVIAGDLFHRSLPS (17) NI--PVF-AVEGNHDK (113) AF---GHIHL
I
II
III
IV

Fig. 2. Alignment of the amino acid sequences of the conserved N-terminal domains of the bacterial SbcD protein and the Mre11 homologs. Similar or identical amino acids are shaded in black. Domains are indicated by numerals I-IV below the sequence alignment. The number of the first residue in each species is indicated at the left. Numbers in parentheses indicate the number of amino acids between each domain. Organisms are as follows: *Ec, Escherichia coli; Hs, Homo sapiens; Sc, Saccharomyces cerevisiae; Sp, Schizosaccharomyces pombe; Ce, Caenorhabditis elegans;* and *Af, Archaeoglobus fulgudis.*

endonuclease activity on single-stranded DNA (ssDNA) and hairpin substrates. Hence the enzymatic activities of MRE11 are entirely consistent with the conservation between the Mre11p homologs and the bacterial nuclease SbcD. MRE11 nuclease activities similarly require Mn^{2+} as a cofactor, but unlike SbcD, MRE11 exonuclease activity does not appear to be ATP-dependent *(23,27,77,109).* 3′–5′ exonuclease activity has been observed on dsDNA templates with a 5′ overhang or blunt ends, but is inhibited by 3′ overhangs greater than 4 bp *(27,77,105,109).* Interestingly, ScMre11p exhibits a slight preference for binding to 3′ overhanging DNA ends, even though its nuclease activity on this substrate is extremely limited *(27,77).* On this basis, it has been speculated that the reduced rate of 5′–3′ resection at DSBs in mutants of the complex may reflect a role for ScMre11p (and by extension hMRE11) in recruiting the *bona fide* 5′–3′ exonuclease(s) to DSB termini in vivo *(77).*

The exonuclease activity of hMRE11 is slightly enhanced by hRAD50 *(77),* but neither the yeast nor the human MRE11 protein requires other complex members for nuclease activity. In contrast to most studies, Trujillo et al. purified the human complex from Raji cells under conditions that left the association of hMRE11, hRAD50, and p95 intact *(105).* They observed 3′–5′ exonuclease activity as with Mrell alone, indicating that hRAD50 and p95 do not alter the polarity of the MRE11 exonuclease.

The Mre11p homologs also exhibit endonuclease activity. This activity is of considerable interest in the context of the V(D)J recombination pathway because of the ability of hMRE11 to cleave a hairpin structure (*77;* Chapter 11). Hairpins are formed on DSBs induced at sites undergoing V(D)J recombination *(57).* Thus the MRE11-RAD50 complex may perform this function in vivo. Suitable mutant cell lines are under development, but a direct assessment of whether these proteins play any role in this process in vivo is not currently feasible. However, recent in vitro results suggest that the hairpin opening activity may be intrinsic to the RAG1 and RAG2 proteins *(7).*

The in vivo significance of the MRE11 nuclease activities is not clearly established, particularly in mitotic cells from yeast and humans. It is perhaps significant, however, that hMRE11 can facilitate NHEJ in vitro. Using linear dsDNA with mismatched 5′ overhanging termini, Paull et al. *(77)* showed that hMRE11-mediated 3′–5′ resection was required for joining of at least one strand of the mismatched termini by DNA ligase I or T4 DNA ligase. These in vitro data do not agree with the studies of Moreau et al. *(69),* who found that a nuclease-deficient *Scmre11* allele did not impair NHEJ in vivo. The extent to which this minimal in vitro reaction reflects the in vivo activity of the complex remains to be seen, but this result suggests a role for the MRE11 3′–5′ exonuclease in DNA endjoining.

4. THE *S. CEREVISIAE* MRE11p-RAD50p-XRS2p PROTEIN COMPLEX

The initial mutagenesis studies of *ScRAD50* revealed most functions of the ScMre11p-ScRad50p-Xrs2p complex *(2).* More recently, the characterization of ScMre11p has expanded our views about the functional significance of this complex in both meiotic and vegetatively growing cells. Mutagenesis of ScMre11p has distinguished particular functional domains of the protein and shed light on its distinct roles in meiotic and mitotic cells.

Null mutants of *ScMRE11, ScRAD50,* and *XRS2* confer essentially identical phenotypes, including IR sensitivity, elevated rates of mitotic interhomolog recombination,

and defects in the initiation of meiotic recombination *(1,2,29,40,62,91).* These data led Kleckner and others to suggest that ScMre11p, ScRad50p, and Xrs2p function in a protein complex *(1,40,84),* which was later confirmed through two-hybrid interaction testing as well as co-immunoprecipitation studies *(44,74,106,109).*

In addition to the three core members of the *S. cerevisiae* complex, genetic and physical evidence for additional members of the complex has come from meiotic cells. In GST "pull down" assays, the ScMre11p C-terminus appears to specifically bind three additional proteins of 40 kDa, 24 kDa, and 22 kDa present in meiotic, but not mitotic extracts *(109).* These proteins have not been identified. However, Usui and colleagues noted that molecular masses of the 24 kDa and 22 kDa species are similar to those (predicted) for Rec102 (23 kDa) and Rec104 (21 kDa), proteins that appear to function in the initiation of meiotic recombination *(8,17).* Further, Xrs2p is phosphorylated in mitotic cells, suggesting that the *S. cerevisiae* complex at least transiently associates with a protein kinase *(109).* In light of these observations as well as the diverse phenotypic outcomes of mutations in the core members of the complex, it is reasonable to assume that the functions of ScMre11p, ScRad50p, and Xrs2p require additional protein interactions. The identification of such interactions will certainly provide important insights regarding the diverse functions of this complex.

4.1. Meiotic Functions of the S. cerevisiae Mre11p-Rad50p-Xrs2p Protein Complex

ScMRE11, ScRAD50, and *XRS2* null mutants exhibit severe meiotic recombination defects. *Scmre11*Δ*, Scrad50*Δ*,* and *xrs2*Δ mutants are blocked at an early point in meiotic recombination, as the formation of viable spores in these mutants requires the *spo13* mutation, which causes the bypass of meiosis I and initiation of meiotic recombination. It was later established that ScMre11p, ScRad50p, and Xrs2p were each required for the formation of DSBs to initiate meiotic recombination *(1,2,19,40,44,51).* Although the formation of DSBs appears to be directly mediated by the Spo11p gene product *(47),* ScMre11p, ScRad50p, and Xrs2p are three of at least nine gene products aside from Spo11p that are required for this event (reviewed in *86*).

The mechanistic basis for the requirement of ScMre11p, ScRad50p, and Xrs2p in the formation of DSBs is not clear. However, certain observations suggest that the complex may influence the formation of "open" chromatin to facilitate cleavage at recombination hotspots. Ohta and colleagues observed that transitions in chromatin structure that normally occur prior to the initiation of meiotic recombination (reviewed in *58*) are affected in mutants of the *S. cerevisiae* Mre11p-Rad50p-Xrs2p complex *(75).* In meiotic cells, localized micrococcal nuclease (MNase) hypersensitive hotspots arise at sites that ultimately correspond to the sites of DSB formation *(58).* These sites are slightly less MNase sensitive in *Scmre11*Δ mutants than in wild-type cells, in contrast to *Scrad50*Δ and *xrs2*Δ mutants in which these sites are significantly more sensitive. In *Scmre11*Δ *Scrad50*Δ double mutants, the increased MNase sensitivity observed in the *Scrad50*Δ single mutant is suppressed to wild-type or slightly lower (i.e., *Scmre11*Δ) levels *(75).* Normal MNase sensitivity is observed in a strain expressing a *Scmre11* allele that lacks nuclease function, *Scmre11D16A* (Table 1). In contrast, reduced MNase sensitivity is conferred by the *Scmre11*Δ*C49* allele, which partially abrogates ScMre11p DNA binding (Table 1). These data suggest that the establishment of appro-

Table 1.
Compendium of *Scmre11* Mutant Alleles

Mutation	Allele name	Interaction with Rad50p	Nuclease activity	Mitotic phenotypes	Meiotic phenotypes	Reference
D16A	*mre11D16A*	ND	Null	Mild MMS sensitivity, short telomeres	DSBs formed but not processed	*(27)*
H18L	*mre11-11*	Weak	ND	Intermediate IR sensitivity	ND	*(15)*
D56N	*mre11D56N*	ND	Null	Weak IR sensitivity	DSBs formed but not processed	*(69)*
D56F	*mre11-2*	No	ND	Null	ND	*(15)*
F58S						
H125N	*mre11H125N*	ND	Null	Weak IR sensitivity	DSBs formed but not processed	*(69)*
H125L	*mre11-3*	Yes	ND	Weak IR sensitivity	Inviable spores	*(14,15)*
D126V						
H213Y	*mre11-58 (mre11S)*	Conflicting data (Subheading 4.5.)	Null	MMS sensitive	DSBs formed but not processed	*(70,106)*
H242L	*mre11-4*	No	ND	Null	ND	*(15)*
H243Y						
ΔAA410-420	*mre11-6*	Yes	Null	Intermediate MMS sensitivity	DSBs formed but not processed	*(109)*
Δ49 AA @ C-terminus	*mre11-ΔC49*	Yes	Wild-type	Wild-type, but does not bind dsDNA	No DSBs formed	*(27)*
Δ62 AA @ C-terminus	*mre11-T10*	Yes	Wild-type	ND	Inviable spores	*(70)*
Δ136 AA @ C-terminus	*mre11-5*	Yes	Wild-type	Wild-type, but does not bind dsDNA	No DSBs formed	*(109)*

ND, not determined.

priate chromatin structure at recombination hotspots requires the DNA binding, but not the nuclease activity of ScMre11p *(27)*.

The *Scmre11S* and *Scrad50S* alleles constitute a class of hypomorphic mutants *(2,19,27,69,70,106)*. Like null *Scmre11* and *Scrad50* mutants, the ***S*** mutants exhibit severe meiotic defects, but differ from the null mutants in two important respects. First, this mutant class is associated with very mild mitotic phenotypes. Second, these alleles do not block the formation of meiotic DSBs, but Spo11p protein remains covalently attached to the DSBs that do form in these strains. Because the protein-DNA adducts remaining at the DSB sites block any subsequent exonucleolytic processing required for DNA recombination, ***S*** mutants produce inviable spores even in *Scmre11S spo13* and *Scrad50S spo13* double mutants *(2,47,48,70)*. The persistent covalent attachment of Spo11p to DSBs formed in the *Scmre11S* and *Scrad50S* mutants suggests a complex of proteins including ScMre11p and ScRad50p cleaves Spo11p following DSB formation. As discussed in Subheadings 4, and 5.5, support for this interpretation comes from the observation that wild-type ScMre11p protein, but not the *Scmre11S* gene products, exhibits nuclease activity in vitro *(69,109)*.

4.2. Mitotic Functions of the S. cerevisiae Mre11p-Rad50p-Xrs2p Protein Complex

4.2.1. Illegitimate Recombination and NHEJ

The phenotypic features of mutants in the ScMre11p-ScRad50p-Xrs2p protein complex in mitotic cells provide a rather dramatic contrast to those in meiotic cells described earlier (Subheading 4.1.). Whereas DNA recombination is profoundly inhibited by mutation of complex members in meiotic cells, mitotic mutants exhibit a hyper-recombinational phenotype. That is, the frequency of spontaneous homologous recombination between heteroalleles in diploid yeast strains is dramatically increased in *Scmre11, Scrad50,* and *xrs2* mutants *(1,40,62)*. On this basis, the ability of mutants to carry out homologous recombination does not appear to be grossly impaired. Mutants of the ScMre11p-ScRad50p-Xrs2p complex are thus distinct from other *ScRAD52* epistasis group mutants such as *Scrad52* and *Scrad51* in which homologous recombination is effectively abolished *(28)*.

Initial clues that ScMre11p, ScRad50p, and Xrs2p function in illegitimate recombination pathways came from plasmid integration/transformation assays. Schiestl and Petes showed that linear DNA lacking any homology to the *S. cerevisiae* genome could nonetheless integrate into the chromosome via short (4 bp) stretches of homology *(90)*. This illegitimate recombination event is not affected by *Scrad52* mutations, but is profoundly impaired in *Scrad50* mutants *(91)*. The ScMre11p-ScRad50p-Xrs2p complex was further implicated in illegitimate recombination by characterization of the yeast NHEJ pathways. Plasmid reclosure assays to define the genetic requirements of NHEJ established the importance of the *S. cerevisiae* Ku70 and Ku80 homologs, DNA ligase IV, and the ScMre11p-ScRad50p-Xrs2p complex in this process *(12,13,67,107,108,115)*. Among those genes, only *Scmre11, Scrad50,* or *xrs2* mutations confer sensitivity to killing by IR or radiomimetic DNA damaging agents *(89,102,115)*. Because NHEJ is impaired to essentially the same extent in these mutants irrespective of their IR sensitivity, it appears that NHEJ per se does not contribute significantly to cellular survival after DSB induction. Further, the profound sensitivity to IR and DSB repair deficiency

observed in *Scmre11*Δ strains therefore argues that defects in the DNA damage response of ScMre11p complex mutants extend beyond NHEJ *(14,15).*

4.2.2. Mitotic Homologous Recombination

An important insight regarding the ScMre11p-ScRad50p-Xrs2p complex in DSB repair came from the observation that the NHEJ deficiency of *Scmre11*Δ mutants is cell-cycle phase-specific. Although the experiments in question specifically focused on NHEJ, the data obtained point to a role of the complex in homologous recombination as well. Moore and Haber found that the ability of *Scmre11*Δ and *Scrad50*Δ mutants to rejoin a chromosomal DSB by NHEJ was much less impaired if the DSB was induced during G1 (three-fold reduction in G1- vs 70-fold reduction in asynchronous cultures). Because overall the ability of the *Scmre11*Δ and *Scrad50*Δ mutants to repair the DSB was profoundly reduced, they inferred that the NHEJ functions of ScMre11p and ScRad50p were restricted to cells in the late S or G2 phase of the cell cycle *(68).* These investigators proposed that the NHEJ defects observed in *Scmre11*Δ and *Scrad50*Δ mutant strains reflect a failure to stabilize and protect the DSB ends from excessive degradation via association with the sister chromatid. Accordingly, their model suggests that the complex plays a role in establishing sister chromatid interactions. The increased rate of spontaneous allelic recombination observed in *Scmre11, Scrad50,* and *xrs2* mutants can also be accounted for by this model if, as a consequence of compromised sister chromatid association, spontaneous lesions normally repaired by sister recombination are repaired by allelic recombination *(68).*

The importance of the ScMre11p-ScRad50p-Xrs2p complex to sister chromatid association and recombination was first suggested by the dose response of *Scrad50* and *xrs2* strains in clonogenic survival assays. Asynchronous cultures of wild-type haploid cells exhibit a biphasic clonogenic survival curve upon irradiation with increasing dose, indicating that relatively sensitive and insensitive populations exist within the asynchronous culture. Because the relatively insensitive population generally corresponds in size to the fraction of cells in G2, its insensitivity is attributed to the presence of a sister chromatid for recombinational DNA repair. Survival curves of haploid *Scrad50* and *xrs2* mutants are not biphasic, suggesting that G2 cells are unable to utilize effectively the sister chromatid for DSB repair *(40,87).* Indeed, more recent analyses of sister chromatid recombination in our laboratory using synchronous cultures as well as chromosomal substrates strongly argue that facilitating the use of the sister chromatid as a template for DSB repair is the primary role of the ScMre11p-ScRad50p-Xrs2p complex in the cellular response to DSBs *(14).* Because sister chromatid interactions in mitotic cells and interhomologue interactions in meiotic cells may share some structural similarities *(52),* it is also noteworthy that *Scrad50* mutants exhibit decreased pairing of chromosomal homologs in meiosis *(113).* Collectively, these data suggest that, in addition to any enzymatic functions it may have, the ScMre11p-ScRad50p-Xrs2p complex plays an important structural role in facilitating chromatid interactions that are critical to DSB repair and the maintenance of chromosome stability.

4.3. Telomeres and Cell-Cycle Checkpoints

An additional manifestation of a structural role for the ScMre11p-ScRad50p-Xrs2p complex may take place at chromosome ends. Several yeast proteins involved in NHEJ are also important for the maintenance of telomeric DNA (*25;* Chapter 14). Abnormal

telomere shortening is observed in mutants of the ScMre11p-ScRad50p-Xrs2p complex, as well as the yeast Ku complex *(11,50,73,82).* In principle, telomere shortening can be attributed to decreased telomerase activity or to telomere degradation, reflecting the abrogation of telomere end protection. Although the specific mechanisms have not been established, analysis of the genetic interactions among telomerase component (*est1* and *est2*), *yku80, Scmre11,* and *Scrad50* mutants has provided some insight. Whereas *yku80* telomere shortening phenotypes are enhanced in *est1* and *est2* mutant backgrounds, this is not the case for *Scmre11* and *Scrad50* mutants. These data suggest that the ScMre11p-ScRad50p-Xrs2p complex is involved in telomere synthesis, and the Ku complex is involved in telomere end protection *(73).*

In light of cell-cycle checkpoint functions mediated by the hMRE11-hRAD50 complex described in Subheading 5.4.1., it is important to consider whether the *S. cerevisiae* complex fulfills a similar role in yeast. This question has not yet been explored in detail, but the evidence available is consistent with a role for the yeast complex in some aspects of cell-cycle checkpoint function. Using a strain in which constitutive expression of the HO endonuclease creates a DSB that must be repaired by NHEJ, Haber and colleagues showed that DSB induction leads to cell-cycle arrest, followed by cell death *(56).* In wild-type cells as well as *Scmre11* and *Scrad50* mutants, cell death was preceded by a limited number of cell divisions as evidenced by the formation of microcolonies. The formation of microcolonies is indicative of escape from, or adaptation to, the cell-cycle checkpoint that is activated in response to the DSB *(78).* Checkpoint adaptation is genetically distinct from checkpoint activation, as casein kinase II and *cdc5* mutants are checkpoint-proficient but adaptation-deficient *(104). yku70 (hdf1)* mutants also undergo cell-cycle arrest followed by cell death, but do not form microcolonies, indicating that these mutants are defective in their ability to adapt to the DSB-induced cell-cycle checkpoint. The adaptation defect of *yku70* mutants is suppressed by *Scmre11*Δ and *Scrad50*Δ mutations *(56),* indicating that the ScMre11p-ScRad50p-Xrs2p complex is required for this aspect of the cell-cycle checkpoint function. The mechanistic basis for this genetic interaction remains unclear. In addition, *Scrad50*Δ mutants are extremely sensitive to hydroxyurea in a manner suggestive of a role for the yeast complex in the activation of the S phase checkpoint *(50).* This facet of the ScMre11p-ScRad50p-Xrs2p complex's function is of great interest. Further examination of DNA damage-dependent cell-cycle checkpoint functions is underway in a number of laboratories. Time will tell whether the linkage of DSB repair to cell-cycle checkpoint functions seen in mammals is a conserved feature of this protein complex.

4.4. Nuclease Activity In Vivo

The complex's impact on checkpoint adaptation appears to correlate with its influence on the 5′-3′ resection at the DSB site *(56).* ScMre11p, ScRad50p, and Xrs2p deficiency also reduces the rate of 5′-3′ resection at the HO-induced DSB at the *MAT* locus during mating-type switching *(34,42,114).* In addition, 5′-3′ resection to facilitate single-strand annealing is slowed by ScRad50p and Xrs2p deficiency *(39,41).* The influence of Mre11p-Rad50p complex members on 5′-3′ end resection is paradoxical, because both yeast and human MRE11 proteins exhibit 3′-5′ exonuclease activity in vitro *(27,69,77,105,109).* It is conceivable that the complex regulates the activity of a *bona fide* 5′-3′ exonuclease or that the in vivo polarity is 5′-3′ as a result of cofactors not

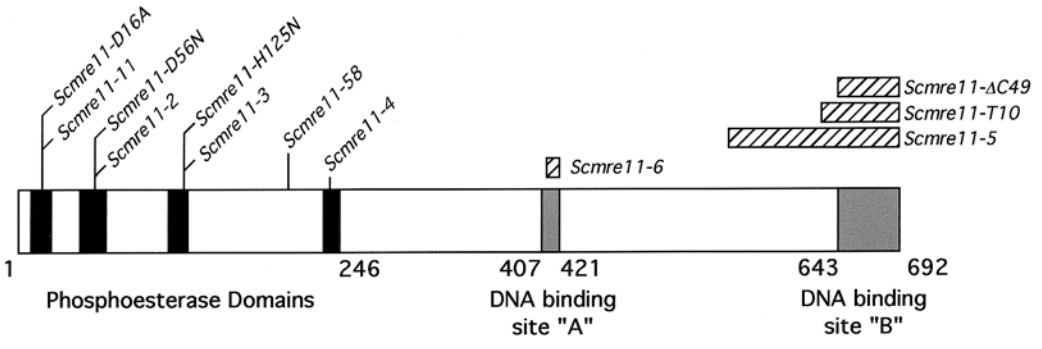


Fig. 3. Mutagenesis of functional domains of *S. cerevisiae* Mre11. Conserved phosphoesterase domains in the N-terminus of ScMre11 are represented by black vertical bars. DNA binding regions in the C-terminus are represented by gray bars. The numbers of the amino acids comprising each functional region are indicated below the diagram. The locations of point mutations in the phosphoesterase domain region are indicated by thin black lines above the diagram. The deletion and truncation mutations in the DNA binding regions are shown by hatched bars. Detailed descriptions of these mutations are given in Subheading 4.5. and Table 1.

present in the in vitro experiments. Alternatively, resection from the DSB site may proceed via the coordinated activities of a DNA helicase and the MRE11 endonuclease *(35,109).* However, on balance it appears very likely that the nuclease activity of MRE11 is not important for its mitotic functions in vivo. This idea is supported by recent data showing that a nuclease-deficient *Scmre11* allele has no impact on DSB resection during mating-type switching, DNA endjoining, or telomere maintenance *(69).*

4.5. Mutational Analyses

Mutagenesis of ScMre11p-ScRad50p-Xrs2p complex members has yielded phenotypic outcomes useful in defining functional domains of these proteins. To a great extent, the primary sequence of the proteins has guided these experiments. Initial mutagenesis targeted the N-terminal ATP binding domain of ScRad50p, generating two classes of point mutations *(2).* The first demonstrated that alteration of ATP binding domain consensus residues is tantamount to a null mutation. The second class, located within this domain but outside of the consensus region, gave rise to the *Scrad50S* mutants. As described earlier (Subheading 4.1.), these mutants exhibit relatively mild mitotic phenotypes and retain the ability to interact with ScMre11p *(16),* yet have severe defects in meiotic recombination *(2).* Mutational analysis to identify functional domains of Xrs2p has not been performed. However, such an investigation may be warranted given the potential analogy of Xrs2p to p95, described in Subheading 5.1.

More recently, mutagenesis of ScMre11p has revealed the protein domains that control nuclease activity, protein interactions, and DNA binding. Deletion and point mutations in the highly conserved N-terminal phosphoesterase domains support the hypothesis that this region of ScMre11p confers nuclease function (Fig. 3 and Table 1). Disruption of the nuclease activity of ScMre11p without affecting the protein's ability to interact with ScRad50p has little impact on mitotic cells, but confers severe meiotic defects, as illustrated by the *Scmre11H125N, Scmre11-3,* and *Scmre11-6* mutants, which

exhibit an ***S*** phenotype (Table 1) *(15,69,109).* As with *Scrad50S* alleles, the spore-inviability phenotype of these nuclease-deficient mutants reflects a defect in the processing of meiotic DSBs owing to the stable covalent attachment of Spo11p to DNA ends.

The heterogeneous phenotypic outcomes of mutations in the phosphoesterase domains of ScMre11p suggest that nuclease deficiency per se is not sufficient to account for the range of phenotypes observed. Because mutations in the first three phosphoesterase domains of ScMre11p *(15,27,69)* alter conserved residues shown to be important for metal-ion binding and catalysis in mammalian and bacteriophage serine/threonine phosphatases *(30,31,120),* one hypothesis is that mutations in these domains disrupt the secondary structure of the protein, thereby affecting other physical interactions required for function. Initial work by H. Ogawa's laboratory identified the N-terminus of ScMre11p as the ScRad50p interaction domain *(44),* and recent studies in our laboratory and others have identified point mutations in this region that disrupt the ScRad50p interaction *(15,70,106).* Impairing the interaction between ScMre11p and ScRad50p results in a null phenotype in mitotic cells, as demonstrated by the *Scmre11-2* and *Scmre11-4* mutants (Table 1) *(15).* The impact of complex disruption on meiotic recombination processes remains uncertain, although the similarities between the *Scmre11D56N* and *Scmre11-2* mutations suggests that the *Scmre11-2* mutant will exhibit a severe meiotic phenotype as well. In addition, there are conflicting data regarding the ability of the Scmre11-58p to interact with ScRad50p *(70,109).* The basis for this discrepancy is not clear, however, its mitotic phenotypes most closely resemble *Scmre11* alleles that lose the ability to interact with the complex.

Intragenic complementation is observed in diploid strains bearing the *Scmre11-5* and *Scmre11-58* mutations *(109)* and in strains bearing the *Scmre11S* and *Scmre11-T10* mutations *(70).* These observations demonstrate the importance of homotypic interactions in the function of the ScMre11p-ScRad50p-Xrs2p complex and also demonstrate that meiotic DSB formation and processing functions of ScMre11p reside in different domains of the protein. Further evidence for this comes from mutational analyses of two regions of ScMre11p important for DNA binding (Fig. 3). Deletion mutations of each of these regions confer marked defects in meiotic recombination (Table 1). Specifically, the *Scmre11-ΔC49* and *Scmre11-5* mutants are defective in meiotic DSB formation *(27,109),* whereas the *Scmre11-6* mutant is deficient in the processing of meiotic DSBs *(109).* Interestingly, the C-terminal region of ScMre11p bearing these DNA binding domains also contains the regions of interaction with meiosis-specific proteins described earlier (Subheading 4.1.), suggesting that ScMre11p may play a role in recruiting proteins to the sites of meiotic DSBs *(109).*

The ScMre11p-ScRad50p-Xrs2p complex has been shown to play a role in diverse aspects of meiotic and mitotic recombination processes. Mutational analyses in *S. cerevisiae* have been critical in the examination of null mutants of protein complex members, as well as in the identification of distinct functional domains of ScMre11p and ScRad50p. Based on the high degree of conservation of these proteins in mammalian cells, these findings may facilitate the design of mutations in mammalian MRE11-RAD50-p95 complex members.

5. THE MAMMALIAN MRE11-RAD50-p95 PROTEIN COMPLEX

The mammalian MRE11, RAD50, and p95 proteins were first identified in human cells as members of a complex that consists of at least four major components, one of which, p400, is currently unidentified (Fig. 1) *(20,26).* hMRE11, hRAD50, and p95 are abundant

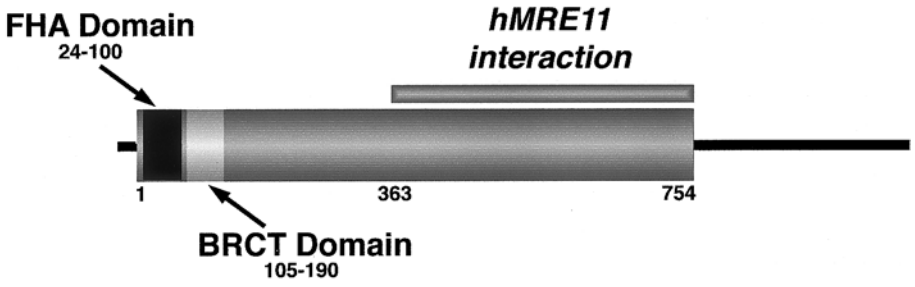


Fig. 4. Structure of the p95 coding sequence. The *NBS1* gene product, p95, was cloned by virtue of its association with the hMre11-hRad50 complex, both by direct protein sequencing from the purified complex and by yeast two-hybrid analyses *(20).* The FHA and BRCT domains, identified by sequence database comparisons, are highlighted; p95 currently is the only protein identified that contains both domains. The portion of p95 identified by yeast two-hybrid interaction with hMre11 is also shown. The thin black lines represent the 5′ and 3′ untranslated regions of the *NBS1* cDNA, and coding portions of the gene are indicated by the thick bar; the numbers below the diagram represent amino acids in the human p95 protein. *NBS1* was identified independently by positional cloning of the gene mutated in Nijmegen breakage syndrome (Subheading 5.4.) *(66,111).*

proteins in a variety of cell lines. Their physical association appears quite stable as all three proteins are co-immunoprecipitated using antisera directed against any one of the three, even in relatively harsh conditions *(20,26).* When purified from HeLa cells, hMRE11, hRAD50, and p95 co-elute in a complex of approx 1.5 MDa molecular mass, indicating that the stoichiometric relationships among members of the complex are not simply one to one. Further, as sizing column fractions corresponding to lower molecular mass do not contain appreciable amounts of hMRE11, hRAD50, or p95, the vast majority of these proteins in the cell are contained in the 1.5 MDa complex. The retention of p400 with the purified complex appears to depend on purification conditions, as this protein does not remain associated under certain chromatographic conditions *(105).* Experiments in vitro as well as in p95-deficient cells demonstrate that hMRE11 interacts directly with hRAD50 and p95, whereas hRAD50 and p95 do not appear to interact directly *(20,77).* Thus hMRE11 appears to be the central molecule in the complex.

5.1. p95: A Divergent Member of the Complex

Highly conserved homologs of *hMRE11* and *hRAD50* are found from yeast to mammals *(26,81).* The product of the *NBS1* gene, p95, appears to have replaced the *S. cerevisiae* Xrs2p protein in the mammalian complex. p95 and Xrs2p exhibit limited similarity in their N-termini, but there is otherwise little or no significant homology between the two. p95 appears to be conserved among mammals, but database searches do not identify strong similarities between p95 and proteins in nonmammalian species *(20).* Conversely, there are no obvious Xrs2p homologs outside of *S. cerevisiae.*

Insofar as both Xrs2p and p95 are stably associated with the Mre11p and Rad50p homologs in their respective species, it is conceivable that the two proteins are functional analogs. The lack of conservation between the two proteins argues against this idea. It is particularly telling that p95, but not Xrs2p, contains a BRCT (BRCA1 C-terminal) domain and a FHA (forkhead-associated) domain at its N-terminus (Fig. 4), both of which are found in proteins involved in DNA repair or cell-cycle control in yeast,

mammals, and other organisms *(10,18).* Interestingly, p95 is the first example of a protein that contains both domains. The phenotypic features of p95-deficient cells discussed later (Subheading 5.4.1.) support the suggestion that p95 function is relevant to DNA damage responses, lending circumstantial evidence for the importance of these conserved protein motifs in p95. Furthermore, *xrs2* and *nbs1* mutant cells in *S. cerevisiae* and humans, respectively, do not exhibit clear similarities in their phenotypic features. A more detailed assessment of Xrs2p and p95 functions is required to determine the extent to which these proteins, and the respective complexes in which they act, are indeed functional analogs.

5.2. Genetic Analyses of the Mammalian MRE11-RAD50-p95 Protein Complex

Genetic analysis of the mammalian MRE11-RAD50-p95 complex has been hampered by the fact that null mutants of *mMre11* and *mRad50* are inviable *(61,116).* Whereas heterozygous *mrad50*Δ/+ mice are normal in all respects, *mrad50*Δ/*mrad50*Δ embryos die at embryonic day 6.5 *(61).* Earlier embryos are not grossly affected, indicating that *mrad50*Δ cells are capable of limited growth. Histological analysis of mutant embryos at day 6.5 suggests that death reflects a gradual failure to proliferate rather than apoptosis *(61).* Day 6.5 of embryogenesis corresponds to the onset of very rapid cellular proliferation *(37),* suggesting that mRAD50 deficiency results in a failure to meet the increased replicative demand.

The bulk of spontaneously occurring DSBs is likely to arise during DNA replication, and available evidence suggests that such breaks are primarily repaired through homologous recombination with the sister chromatid *(24,54).* As described earlier (Subheading 4.2.2.), several lines of evidence indicate that *S. cerevisiae* Mre11p-Rad50p-Xrs2p complex mutants exhibit defects in their ability to utilize the sister chromatid as a template for DSB repair. If sister chromatid-based repair of such spontaneously arising DSBs were impaired by loss of mRAD50, *mrad50*Δ/*mrad50*Δ cells undergoing rapid proliferation would be disproportionately affected. Hence, the proliferative failure observed could be attributed to the rapid accumulation of unrepaired or misrepaired spontaneous DSBs during the normally rapid cell division that occurs at this stage. Experiments using a conditional *Gdmre11* mutant in the DT40 chicken cell line support this interpretation. Following inactivation of the *GdMRE11* gene, these cells proceed through several cell cycles before death. Karyotypic analysis during this process reveals that chromosomal aberrations begin to accumulate by 72 h after GdMRE11 depletion *(117).* These observations underscore the importance of the mammalian MRE11-RAD50-p95 complex specifically, and of DNA recombination functions generally, in normal cell growth.

Lethality of the *mmre11* and *mrad50* null mutations has precluded detailed analysis of recombinational DNA repair functions of those gene products. However, cultured *mrad50*Δ/*mrad50*Δ blastocysts are highly sensitive to killing by IR *(61).* Hence, it appears that mRAD50 deficiency leads to DSB repair deficiency, confirming that *mRad50* functions in the DNA damage response of murine cells as predicted from the phenotypic features of *Scrad50* mutant strains.

5.3. Cytology of the hMRE11-hRAD50-p95 Protein Complex

The hMRE11-hRAD50-p95 complex's role in the mammalian DSB repair response was revealed by cytological examination of its subcellular localization in human fibrob-

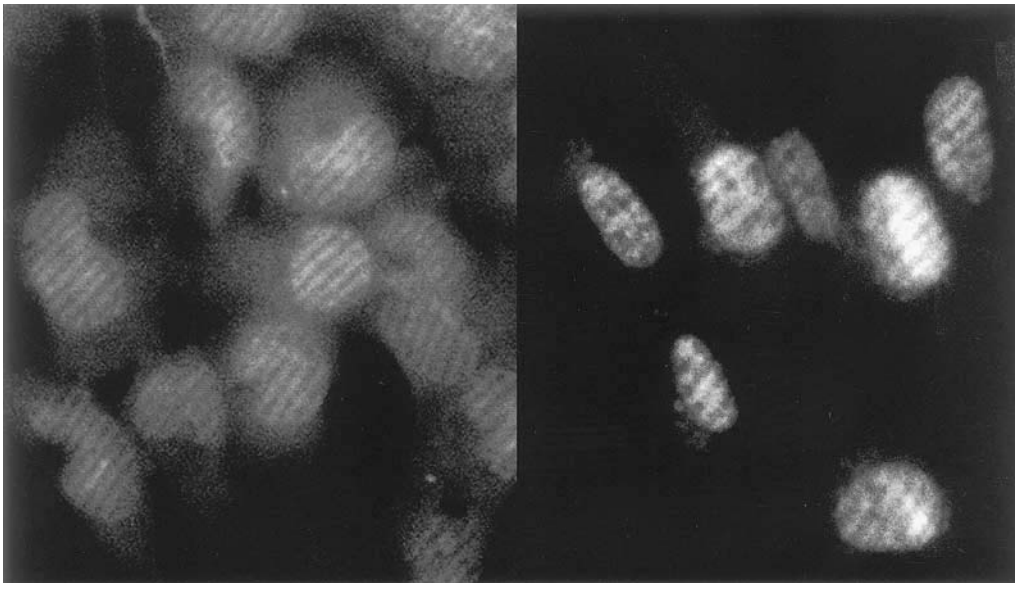


Fig. 5. The hMre11-hRad50-p95 protein complex localizes to DNA damage. Human fibroblasts were irradiated with ultrasoft X-rays through a gold grid *(71).* Left panel: At 30 min after irradiation, cells were labeled for DNA DSBs with BrdU. The DSBs are observed in the same stripe pattern as imposed by the presence of the gold grid. The DSBs persist in this pattern until they are repaired. Right panel: Identically irradiated cells were stained for the hMre11-hRad50-p95 protein complex with hMre11 antiserum. The complex redistributes into the same striped pattern as DNA DSBs, until DSB repair is complete. Independent determinations revealed that stripes of DSBs and hMre11 were identical, demonstrating that localization to DSBs is an important facet of the hMre11-hRad50-p95 complex's role in the cellular DNA damage response *(71).*

lasts. The hMRE11-hRAD50-p95 complex is homogeneously distributed in the nucleus, as shown by immunofluorescence with antisera directed against each of the complex members *(20,64).* However, upon the induction of DNA DSBs, the complex becomes associated with DSBs, and remains at DSB sites until the damage is repaired. These experiments have also yielded insight regarding the temporal and spatial behavior of the hMRE11-hRAD50-p95 complex with respect to other mammalian DSB repair proteins. Because p95 deficiency abrogates a specific cell-cycle checkpoint that functions in response to DNA damage (Subheading 5.4.1.), association of the hMRE11-hRAD50-p95 complex with DSBs suggests that the DNA-damage recognition functions of the complex are linked to the signal-transduction pathway(s) required to activate cell-cycle checkpoints.

5.3.1. Partial Volume Irradiation

Visualization of the hMRE11-hRAD50-p95 complex at DSBs required specialized techniques to induce and detect DNA DSBs in discrete subnuclear volumes. Taking advantage of the properties of ultrasoft X-rays to create DNA damage within a spatial range of 50 nm or less, Nelms et al. *(71)* induced DSBs within defined domains of fibroblast nuclei by irradiating cells through a special "striped" gold grid, a technique called partial volume irradiation. The DSBs, labeled fluorescently at dsDNA ends, were

subsequently observed in the striped pattern imposed by the grid mask soon after irradiation (20–30 min) (Fig. 5) *(71).* Whereas DSBs were no longer detectable by this method 90 min after irradiation in repair-proficient cells, the damage persisted in the striped pattern for up to 5 h after DSB induction in the human DSB repair-deficient cell line, 180BR (deficient in DNA ligase IV *[85]*). Independent determination of DSB repair kinetics show that the disappearance of stripes coincides with the completion (or lack) of DSB repair *(64,71).* These observations provided surprising but compelling evidence that the vast majority of DSBs remain stationary within the nucleus of irradiated cells, and thereby revealed an implicit requirement that cells possess a diffusible "sensor" of DNA damage to activate the cellular DNA damage response.

The hMRE11-hRAD50-p95 complex becomes associated with DSBs relatively early after their induction. Co-immunofluorescence with hMRE11 antiserum showed that the hMRE11-hRAD50-p95 complex had re-localized from its normal homogeneous nuclear distribution to the same "stripes" of DNA DSBs, and remained localized until the damage was repaired (Fig. 5) *(71).* In human DSB repair-deficient 180BR cells, hMRE11 remained associated with DSBs for the 5 h in which DSBs remained unrepaired and confined to stripes. This study thus demonstrated that an important spatial component of DSB repair is the localization of DSB repair proteins to the sites of DSBs, rather than the movement of DNA damage to fixed domains of repair complexes. The localization of hMRE11-hRAD50-p95 at DSBs is reflective of this complex's role in DSB repair and response pathways. Combined with the inability of p95-deficient cells to activate a DNA damage checkpoint pathway (Subheading 5.4.1.), these data suggest that the complex functions early in the mammalian DSB response and is situated to act either as a molecular sensor of DNA damage or in close conjunction with such sensors. However, neither the mechanism by which the hMRE11-hRAD50-p95 complex comes into contact with DNA DSBs nor the nature of the signal elicited by their association with DSBs has been defined.

5.3.2. Ionizing Radiation-Induced Foci

Experiments utilizing conventional "hard" X-rays, in which IR-induced DNA damage is uniformly distributed, have also proven useful in defining the role of the hMRE11-hRAD50-p95 complex in mammalian DSB responses. Upon γ-irradiation, the complex redistributes into focal nuclear structures, termed ionizing radiation-induced foci (IRIF) *(64).* Immunofluorescence co-localization experiments have demonstrated that IRIF contain each of the complex members *(20,64),* providing further evidence that hMRE11, hRAD50, and p95 act in a complex during the cellular response to DNA damage.

A number of observations support the idea that IRIF formation reflects the function(s) of the hMRE11-hRAD50-p95 complex in the response to DSB induction. First, hMRE11-hRAD50-p95 IRIF are dependent on the prior induction of DSBs, and form in a dose-dependent manner *(64);* IRIF are not induced by other types of DNA damage. Second, hMRE11-hRAD50-p95 IRIF formation is dependent on the genetic background of the cells examined. The DSB repair-deficient human cell line, 180BR, exhibits increased IRIF formation at equivalent X-ray doses compared to normal repair-proficient cells *(64),* consistent with the interpretation that IRIF multiplicity is a function of the number of DSBs. hMRE11 and hRAD50 IRIF are not detected in p95-deficient human cells (cells from patients with NBS; Subheading 5.4.), which

reveals that although p95 is not important for hMRE11-hRAD50 interaction *(20),* it is important for this aspect of the complex's function. hMRE11 and hRAD50 IRIF formation is also profoundly reduced in SV40-transformed A-T mutant cell lines when compared to normal SV40-transformed cells *(64).* The hMRE11-hRAD50 IRIF response is reduced to a lesser extent in primary (non-SV40-transformed) A-T cells *(63),* indicating that SV40 transformation affects the behavior of the complex. Conversely, the lack of DNA-PK_{cs} or p53 had no effect on IRIF formation *(64),* suggesting that although these proteins also have roles in cellular DSB responses, their functions are independent of hMRE11, hRAD50, and p95.

These cytological assays have proven useful in assessing the relative functions of the hMRE11-hRAD50-p95 complex and hRad51, a protein that mediates DNA strand exchange *(5,32).* hRad51 forms foci following DNA damage, and also during S phase in un-irradiated cells *(33,101).* Interestingly, hRad51 nuclear foci also appear to contain the BRCA1 and BRCA2 proteins, supporting a role for these tumor-suppressor proteins in recombinational DNA repair (*21,92;* Chapter 10). hMRE11-hRAD50-p95 IRIF do not co-localize with hRad51 foci, nor are they coincident within the same nucleus *(64).* SV40-transformed A-T cells, which demonstrated reduced hMRE11-hRAD50 IRIF formation, exhibited markedly increased numbers of cells with hRad51 foci *(64).* The formation of both hMRE11 (hRAD50) and hRad51 foci is induced by DNA damage. The exclusivity of hMRE11-hRAD50-p95 complex IRIF and those of hRad51 presumably reflects the differing spatial and temporal requirements for these protein complexes in DSB repair. Hence, these cytological data are consistent with the prevailing models of DSB repair that situate the functions of Rad51 and the MRE11-RAD50-p95 complex at distinct points in the DSB repair process. Further experiments using these cytological assays will be useful in the analysis of the DSB response of various mutants of the hMRE11-hRAD50-p95 complex.

5.4. Clinical Considerations

The genomic instability resulting from disruption of the ScMre11p-ScRad50p-Xrs2p complex bears some resemblance to the cellular features of chromosome instability syndromes in humans. Thus it had been suggested that congenital or somatically acquired deficiencies in hMRE11-hRAD50-p95 complex members might be causative of such instability in human cells *(80).* Unlike yeast, null mutations in *mMre11* and *mRad50* are lethal in the mouse *(61,116);* null mutations in either are presumably lethal in humans as well. However, the hypothesis that human genetic instability can be caused by disrupting the hMRE11-hRAD50-p95 complex has been validated by the discovery that *NBS1* mutations cause NBS, a hereditary chromosome instability disorder *(20,66,111).* This extremely rare disease is characterized by developmental defects and a predisposition to malignancy (reviewed in *110*). At the cellular level, peripheral lymphocytes from NBS patients harbor characteristic chromosomal rearrangements. NBS cell lines are sensitive to IR and exhibit radioresistant DNA synthesis (RDS). These cellular features are similar to those of cell lines derived from patients with A-T, another chromosome instability and cancer-prone syndrome *(95).* NBS had been described as an A-T variant syndrome until it became clear that they were genetically separable *(95).* Nearly all NBS patients described to date are homozygous for the same mutant allele of the *NBS1* gene, a frameshift mutation predicted to truncate greater than

two-thirds of the C-terminus from p95 *(111)*. The cellular and clinical sequelae of p95 deficiency underscore the importance of the hMRE11-hRAD50-p95 complex in the maintenance of genomic integrity. An additional demonstration of human genomic instability caused by Mre11-Rad50-p95 deficiency has recently been documented by the identification of *hMre11* mutations in another A-T like syndrome *(98)*.

5.4.1. Cellular Phenotypes Associated With NBS

The cellular defects associated with p95 deficiency have revealed much about how the hMRE11-hRAD50-p95 complex may integrate the DSB repair response with cell-cycle control. First, NBS cell lines are sensitive to IR and radiomimetics, and display highly elevated numbers of chromosome aberrations following IR *(4,72,99)*. In spite of p95's association with hMRE11 and hRAD50, physical analysis of DSB repair in NBS cells does not indicate a profound defect in DSB rejoining *(72)*. The most significant defect in the DNA damage response of p95-deficient cell lines is the failure to arrest DNA synthesis in response to IR. This defect in S-phase regulation is also a hallmark of A-T cells. However, unlike A-T cells, NBS cells appear to retain the ability to arrest in G1 and G2, and do not share the profound deficiency in p53 induction following IR *(45,65,118)*. Characterization of the S-phase checkpoint defects in A-T cells and *S. cerevisiae mec1* mutants suggests that this mode of cell-cycle regulation is manifest in the inhibition of replication origin firing *(76,88,97)*. Similar analyses have not been carried out in NBS cells. Understanding the molecular biology of this S-phase checkpoint will be crucial to discerning the role of the hMRE11-hRAD50-p95 complex in its activation.

The cellular defects that result from a lack of p95, in combination with the previously described cytological, genetic, and biochemical data, clearly demonstrate that the MRE11-RAD50-p95 complex is an important mediator of the cellular DNA DSB response. The hMRE11-hRAD50-p95 complex is uniquely situated in this DNA damage response by the linkage of DNA repair proteins to a molecule that functions in an S-phase checkpoint. The hMRE11-hRAD50-p95 complex localizes to DNA DSBs (Subheading 5.3.); however, in the absence of p95, cells fail to respond appropriately to the presence of DSBs. Thus, it is conceivable that the hMRE11-hRAD50-p95 complex functions in the recognition of DNA DSBs or in close concert with the sensors of such damage. The phenotypic features of NBS cells further indicate that the complex is important for the activation of the S-phase cell-cycle checkpoint. The molecular basis of cell-cycle checkpoint activation by the complex is not understood. This unresolved question remains one of the most critical issues in understanding the integration of the MRE11-RAD50-p95 complex's functions within the cellular DNA damage response.

5.4.2. Clinical Manifestations of p95 Deficiency

The clinical findings associated with p95 deficiency are represented by patients with NBS. However, the syndrome is extremely rare—fewer than 100 patients have been described to date—and patients exhibit some clinical variability. The diminutive size and microcephaly of the patients are the most common and prominent outward manifestations, and mental retardation has been noted for certain patients. NBS patients also display a strong predisposition to malignancy. Of 42 patients in the NBS registry as of 1996, 15 patients (between 1 and 22 yr of age) had developed malignant tumors; 80% of these were lymphomas *(110)*. Chromosome rearrangements in peripheral lymphocytes are common and typically involve regions on chromosomes 7 and 14 at which antigen recep-

tor loci undergo programmed gene rearrangement. Certain immunoglobulin isotype deficiencies have also been reported. Although NBS is a recessive disorder, heterozygotes may exhibit some of the phenotypes, especially predisposition to cancer *(93).* A more detailed review of clinical findings in NBS patients can be found elsewhere *(110).*

5.4.3. Clinical Manifestations of hMre11 Mutations

The recent identification of non-null mutations in *hMre11* that result in an A-T like disorder (termed ATLD) *(98)* further validates the hypothesis that mutations affecting the Mre11-Rad50-p95 complex lead to genomic instability. The phenotypes of these patients were puzzling in that they exhibited many of the clinical hallmarks of A-T, but mutations in the *ATM* gene were not detected *(36,98).* The finding of hypomorphic *hmre11* alleles was a suprising result because these patients do not share clinical phenotypes typically associated with NBS. However, cell lines derived from ATLD patients more closely resemble NBS cells with respect to radiosensitivity, RDS, and p53 induction, than classical A-T cells *(98).* The phenotypes represented by ATLD and NBS strongly suggest that the functions of the hMRE11-hRAD50-p95 complex and ATM overlap to some extent. However, a more complete analysis of these functions in mammals awaits the creation of genetically defined mouse models of MRE11, p95, and RAD50 deficiency.

6. TOWARD A MODEL FOR THE FUNCTION OF THE MRE11-RAD50-p95 PROTEIN COMPLEX IN THE CELLULAR RESPONSE TO DNA DAMAGE

The diverse phenotypic outcomes imparted by deficiency in members of the MRE11-RAD50-p95 (Xrs2p) complex preclude a simple unifying model for its function. Although our current picture of this complex has been assembled from data obtained in both yeast and mammals, the extraordinary conservation of MRE11 and RAD50 suggests that many functions of the complex are likely to be conserved. The pleiotropy of mutations affecting the complex situate it at the heart of the cellular response to DSBs (Fig. 6). From this perspective, elucidation of the genetic and physical interactions that link this complex to the network of functions that constitute the cellular response to DSBs is a crucial next step. A clear molecular definition of the in vivo enzymatic functions of the MRE11-RAD50-p95 (Xrs2p) complex will also provide important insight to its direct roles in the DSB response.

Its impact on sister chromatid recombination and chromatin structure suggest that the MRE11-RAD50-p95 (Xrs2p) complex plays an important structural, as opposed to an enzymatic role in aspects of DNA recombination and repair. Whereas this interpretation is supported by genetic analysis in *S. cerevisiae,* the abundance of the human MRE11-RAD50-p95 complex similarly argues against a purely enzymatic role in human cells *(20,26).* The telomere maintenance functions of the complex may also reflect such a structural role, because the complex does not appear to function in telomere end protection nor does it directly influence telomerase activity *(73).* Mechanistic information regarding the telomere-shortening phenotype of *S. cerevisiae* mutants and determination of whether the mammalian complex also functions at telomeres are important to this issue.

The abrogation of S-phase checkpoint activation in p95-deficient cells is one of several lines of evidence supporting the idea that the complex also mediates regulatory functions in the cellular response to DSBs. These regulatory functions presumably follow from the

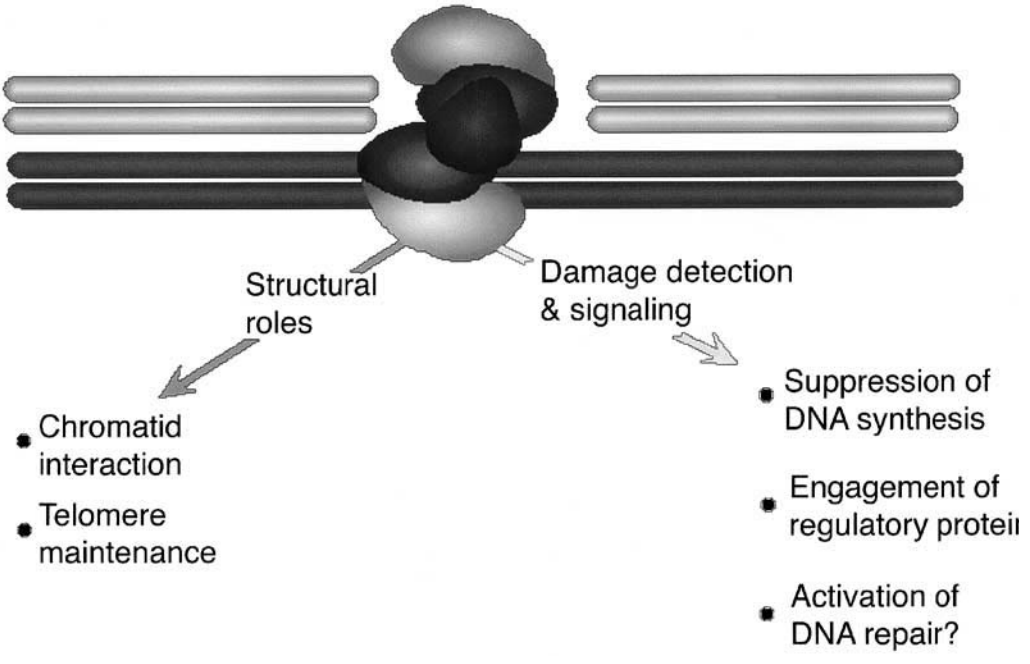


Fig. 6. Functions of the Mre11-Rad50-p95 (Xrs2p) protein complex in the DSB response. Based on cytological evidence, we hypothesize that the complex is situated at or near the site(s) of DSBs. Once at the site, the complex plays a structural role in facilitating the appropriate template utilization during the DSB repair process. Other structural roles for the complex may include its role in telomere maintenance. The phenotypic features of NBS cells indicate that once at the site of DNA damage, the complex is important for activating cell-cycle checkpoint responses, particularly those that lead to the suppression of DNA synthesis. Presumably, its role in cell-cycle checkpoint functions requires interaction between the complex and as yet unidentified regulatory proteins. Finally, we speculate that the signaling function of the complex may also lead to the activation of DNA repair, although evidence for this has not been established.

complex's DNA damage-recognition functions. What are the molecules downstream of the DNA damage recognition event? What is the molecular nature of the interaction between these molecules and the MRE11-RAD50-p95 complex? It is certainly clear that the complex interacts, either directly or indirectly, with proteins that mediate the S-phase checkpoint in human cells. Molecular characterization of this interaction will provide fundamental insight regarding the mechanisms of cell-cycle checkpoint activation.

Even in the absence of a comprehensive model for the complex's function, it is clear that MRE11, RAD50, and p95 (Xrs2p) play a fundamental role in the maintenance of genomic integrity. In the vast network of biochemical functions that must be integrated into the cell's response to genotoxic stress, the MRE11-RAD50-p95 (Xrs2p) complex occupies a critical hub at the interface of DNA repair pathways and the activation of cell-cycle checkpoints.

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8

Repair of DNA Double-Strand Breaks and Mismatches in *Drosophila*

Carlos C. Flores

1. SCOPE

This review focuses on recent progress in the study of long-patch mismatch repair (MMR) and double-strand break repair (DSBR) in *Drosophila melanogaster.* Some excellent reviews that overlap these subjects have been published recently *(52,64,92,154).* A web site summarizing the DNA repair genes of *Drosophila* has been assembled *(24).* Also, FlyBase *(45)* and the Berkeley *Drosophila* Genome Project (BDGP) *(6)* are superb resources for information on most aspects of Drosophila research. For comprehensive reviews on how meiotic recombination is intertwined with the progression of oocyte development, *see* Morris and Lehmann *(116)* and Gonzalez-Reyes *(56).* Insightful reviews of other types of DNA repair in Drosophila have also been published *(39,64,92,124,154).*

2. OVERVIEW

Historically, *Drosophila* research has greatly increased our knowledge of DNA repair and recombination. For example, pioneering studies of the effects of X-rays on chromosomes *(118)* and the detailed cytogenetics of meiotic recombination *(26)* were performed in fruitflies. *Drosophila* research in general, and DNA repair studies in particular, are poised for a renaissance. The publicly funded genome projects (primarily BDGP), in conjunction with Celera, announced that the genome sequence is nearly complete *(1).* This project also produced a wealth of accompanying resources: thousands of cDNAs, genomic clones, and P element insertion lines. A promising new gene-targeting method was developed, which not only may make it easier to mutate genes at will, but also may become a valuable additional tool to investigate recombinational repair *(141).* Techniques to study the repair of DNA injected into embryos have been refined. And with the accumulation of nearly a century of data, mutants, and genetic tools, such as deletions and balancer chromosomes, along with the ease of mutational screens, and methods for producing somatic clones, *Drosophila* remains an unparalleled genetic powerhouse.

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In the past decade studies in yeast and mice have dominated in the accelerating field of DNA repair. So what has *Drosophila* contributed to this juggernaut? Flies have furnished several components including some strange surprises. For example, *Drosophila* has fueled the re-evaluation of homologous recombination (HR) mechanisms that do not rely on Holliday-junction cleavage *(120).* Studies in *Drosophila* also produced a curious mystery: during repair of a DSB, there can be a cis-bias in the use of repair templates that extends over many megabases *(41).* It is puzzling how co-linearity can be evaluated over such a long linear distance within the relatively small nucleus. Flies were also first to furnish an interesting link between nucleotide-excision repair (NER), MMR, and meiotic recombination, by analysis of the *mei-9* gene *(155).* Other Drosophila studies have uncovered an association between regulation of oocyte development and meiotic recombination involving spindle-class genes with roles in DSBR *(49).*

3. MISMATCH REPAIR IN DROSOPHILA

3.1. Early Steps of Mismatch Repair

Mismatched bases can arise in the DNA duplex by several mechanisms, though probably they most often occur as a result of DNA replication errors. In all organisms a major pathway to repair these errors is the long-patch MMR system. MMR is studied in humans and several model organisms but is most thoroughly understood in *Escherichia coli* and secondly *Saccharomyces cerevisiae.* In *E. coli,* base-pair mismatches and small insertions or deletions are recognized by a homodimer of MutS protein. MutS binds to these structures and recruits other proteins, including MutL, needed to resolve the contradiction. The complex can identify which strand to repair by sensing the methylation state of both strands. A region of up to a few kilobases is removed from the "mutant" (under-methylated) strand by exonuclease activity. DNA polymerase then replaces the sequence, thereby resolving the mismatch (reviewed in *22,140*). In all organisms this process employs homologs of the *E. coli mutS* and *mutL* genes *(22,32,85,100).*

In eukaryotes the method of strand discrimination is still largely a mystery. The most popular explanation is that a pre-existing nick is usually present and that the broken strand is targeted for degradation and correction. The most commonly considered sites of MMR are newly replicated DNA and HR intermediates. Pre-existing nicks would be present near the replication fork and are strongly predicted in Holliday structures and newly resolved Holliday structures of recombination intermediates. But mismatches can also arise through chemical damage to DNA, and it is not clear whether there is a nick-independent method of strand discrimination for long-patch MMR.

Replication can give rise to mismatches either by misincorporation or by polymerase slippage in regions of sequence repeats. Extensive repeats of very short DNA sequences (microsatellites) are highly vulnerable to mutation in the absence of MMR *(158,163).* Changes in the number of repeats are easily detected by analyzing the size of polymerase chain reaction (PCR) products. Through this hallmark of repeat instability, MMR was discovered to be important in preventing human cancer. People who inherit a mutation in the *mutS* or *mutL* homologs *hMSH2, hMLH1,* (and to a lesser degree, *hMSH6, hPMS1,* or *hPMS2*) have a greatly increased risk of certain cancers, especially hereditary nonpolyposis colon cancer (HNPCC) *(42,95,123,132).* Simple DNA repeats are extremely unstable in the cells of these cancers and in some sporadic cancers (reviewed in *22,103,139).*

Table 1
MutS and MutH Homologs Identified in Yeast, Humans, and Drosophila

S. cerevisiae[a]	Function[b]	H. sapiens[c]	*D. melanogaster*[c]
MSH1	9	?	None
MSH2	1, 2, 3, 6, 7, 8, 10	*hMSH2* 40% (966 a.a.)	*spel1* 35% (909 a.a.)
MSH3	3, 6, 7, 8, 11	*Dupl (hMSH3)* 34% (993 a.a.)	None
MSH4	6, 7, 8, 12	*hMSH4* 32% (776 a.a.)	None
MSH5	6, 7, 12	*hMSH5* 29% (650 a.a.)	None
MSH6	1, 2, 6, 11	*hMSH6* 32% (1172 a.a.)	*Dmmsh6* 25% (1044 a.a.)
MLH1	1, 2, 5, 8, 13	*hMLH1* 36% (787 a.a.)	*Dmmlh1* 35% (771 a.a.)
PMS1	1, 2, 3, 6, 14	*hPMS1* 30% (336a.a)	None
?	?	*hPMS2* 33% (904 a.a.)	*Dmpms2* 33% (929 a.a.)
MLH2	8, 14	?	None
MLH3	3, 4, 5, 14	*hMLH3* 24% (374a.a.)	None

[a] Name of *S. cerevisiae* gene.

[b] Function of characterized yeast and/or human protein. 1, Repair of base:base mismatches; 2, Repair of small-loop mismatches; 3, Repair of larger-loop mismatches; 4, Required for normal levels of meiotic recombination, 5, Required for normal levels of meiotic reciprocal exchange; 6, Required for heteroduplex rejection; 7, Required for trimming nonhomologous single-stranded ends; 8, Required for resistance to certain DNA damaging agents; 9, Mitochondrial DNA MMR; 10, Forms dimer with either MSH3 or MSH6; 11, Forms dimer with MSH2; 12, Forms an MSH4/MSH5 dimer; 13, Forms dimers with PMS1, MLH2, and MLH3; 14, Forms dimer with MLH1.

[c] Name of homologous gene/percent amino acid identity (length of aligned region). ?: no homolog identified; None: apparently no homolog exists. Alignments were performed by the algorithm of Altschul et al. *(2a)* with the BLOSUM62 identity matrix and gap penalty of 11, gap extension penalty of 1.

3.2. Drosophila MMR Genes

The MMR system of Drosophila appears less complex than either the human or yeast system. Certainly, the contingent of recognizable MMR genes is smaller in flies. Although *S. cerevisiae* has at least six MutS homologs *(MSH1-6),* and at least five exist in humans, only two MSH genes have been discovered in flies. Also, *Drosophila* seems to have only two MutL homologs (MLH) compared to four in yeast, and four or more in humans (Table 1). This is somewhat similar to the case in the nematode, *Caenorhabditis elegans,* but it is curious that the yeast system correlates more closely to humans. Even more surprising is the apparent absence of the meiosis-specific MSH genes in Drosophila (*MSH4* and *MSH5*) that are present in yeast, nematode, and human. Although some MSH and MLH genes of yeast and humans appear to be partially redundant for particular phenotypes, none are completely redundant. The absence of MSH1, MSH3, MSH4, MSH5, and MLH2 and MLH3 in flies can be rationalized in at least three ways: (1) Drosophila does not require the specialized functions of the missing genes; (2) unrelated proteins provide those specialized functions; or (3) the genes extant in Drosophila produce broader activities that encompass the specialized functions (the production of multiple proteins from a single gene may also play a role). Some combination of the latter two possibilities may be the most likely scenario.

Three Drosophila MMR genes have been cloned using sequence similarity: two MLH genes, *Dmlh1* and *Dpms2,* and the MSH gene *spellchecker1 (spel1).* More recently, an *msh6* gene has been identified within the sequence produced by the BDGP.

These genes that participate in the earliest steps of MMR are highly conserved and easy to identify by sequence similarity alone. The other genes involved in MMR can be less obvious because they include more generally employed DNA helicases, SS-exonucleases, DNA polymerase, and DNA ligase.

3.2.1. spel1

The *spellchecker1* gene was isolated by virtue of its similarity to other MSH genes using degenerate PCR. Its sequence indicates that it is a member of the MSH2 branch of the family. In yeast and humans, *msh2* is required for virtually all MMR in the nucleus. Null *spel1* mutant Drosophila were assembled using a pair of chromosomes bearing unique, large deletions. The deletions only overlap in a small region containing *spel1* and an adjacent gene known as *lethal(2)35Aa.* Because the adjacent gene is required for survival, a cloned copy was supplied as a transgene *(43).*

The *spel1* mutants created in this way are viable and fertile. DSBR does not appear grossly hindered in *spel1* mutants because they are not significantly more sensitive to γ-irradiation. Neither are they affected in their sensitivity to methylmethane sulfonate (MMS) *(43).* In contrast, *msh2* mutant human cancer cell lines and mouse cells have an increased tolerance to simple methylating agents *(19,23).* Presumably, in the mammalian case, the intact MMR system recognizes the damaged DNA but cannot complete repair. Instead it converts the methylated bases into a lesion that is more lethal and/or transmits a signal leading to cell-cycle arrest and apoptosis.

In *E. coli,* yeast and humans, short DNA repeats (microsatellites) are highly vulnerable to mutation in the absence of MMR *(42,158,163).* Microsatellites are also very unstable in *spel1* mutant flies. Chromosomes were analyzed after passage through 12 fly generations without SPEL1. One of the most unstable microsatellite loci analyzed had detectable mutations in over 25% of the tested chromosomes. No mutations were detected at that same locus in a hemizygous $spel1^{+/-}$ background (0 out of 192 chromosomes) *(43).* Loss of SPEL1 also destabilizes microsatellite repeats during the process of gene conversion. In this experiment, gene conversion was triggered by induction of a DSB in the parental germline. The products of conversion were recovered in the progeny and their structure was analyzed. The fidelity of copying a dinucleotide repeat from the homolog was fivefold higher in controls than in *spel1* mutants *(43).*

3.2.2. Dmmlh1 and Dmpms2

Drosophila homologs of *MLH1* and *PMS2* genes have been cloned and sequenced. Their genomic locations have also been identified, but as yet no mutants are known to exist. *Dmlh1* mRNA is abundant in ovaries and embryos and is present at lower levels in later stages. *Dpms2* mRNA appears to be expressed more uniformly throughout development. Curiously, *Dpms2* appears to have sex-specific transcriptional start-points or differential splicing such that a 5′ segment is found in transcripts from male larvae and male adults but not from females *(18,108,109,160).*

3.2.3. Tosca

The *tosca (tos)* gene encodes a putative exonuclease in flies *(34).* Its amino acid sequence establishes it as a member the XPG/Rad2 superfamily of endo/exonucleases. It is sufficiently related to *S. pombe,* mouse, and human EXO1 that it is probably the Drosophila *exo1* homolog. However, it appears that the expression of *tos* is completely

restricted to the female germline and early embryo. Early in oogenesis, *tos* mRNA is enriched in the pro-oocyte at a time that coincides with pachytene, implying that TOS may play a role in meiotic recombination. (Note that meiotic crossing-over occurs only in female Drosophila, not in males.) Later, *tos* mRNA is very abundant during the extremely rapid nuclear divisions of the early embryo, suggesting an additional role during this extraordinary DNA replication. MMR has been proposed as a possible role of TOS at this stage *(34).* This notion is supported by evidence that *exo1* of *S. pombe* has a role in MMR as demonstrated by genetic analysis *(146),* and the human EXO1 protein binds to MSH2 *(150).* Murine *exo1* is highly expressed in the testes and is especially abundant during the stage at which meiotic recombination occurs *(96).* Levels of EXO1 are also high in the lymphoid tissues of the mouse, especially the spleen. If *tosca* is the main exonuclease of MMR in flies, it is hard to explain why its expression is undetectable after early embryonic development and absent in males. It is possible that TOS has a partially redundant role in MMR.

3.2.4. mei-9

On the surface, MMR in flies may seem to fit the simple paradigm worked out in *E. coli,* with a few minor modifications, e.g., SPEL1/MSH6 heterodimer replacing MutS, and a MLH1/PMS2 heterodimer replacing MutL. However, it is likely to be more complex, as illustrated by *mei-9.* The *mei-9* gene was first identified almost 30 years ago in a screen for mutants defective in meiotic recombination. It was originally found that mutations in *mei-9* reduced meiotic crossing-over to less than 10% of the normal level *(3).* Later, *mei-9* mutants were isolated in screens for mutagen sensitivity. Through these studies it was discovered that they were very hypersensitive to ionizing radiation, MMS, nitrogen mustard, 2-acetylaminofluorence (AAF), and ultraviolet (UV) irradiation *(15,151).* Furthermore, *mei-9* mutants were found to be deficient in the repair of UV-induced pyrimidine dimers *(38).* This block in NER proved to be at some stage before strand cleavage *(4,15).*

Meanwhile, evidence that *mei-9* might also be involved in MMR surfaced when it was discovered that even though the frequency of meiotic gene conversion was unaffected, *mei-9* mutants exhibited postmeiotic segregation (PMS) *(28).* During meiotic recombination, heteroduplex is formed at the sites of exchange. Normally it is repaired before mature gametes are formed, but any heteroduplex that fails to be corrected will generate mosaic offspring. With PMS, the two maternal (or two paternal) alleles represented in the heteroduplex begin segregating at the first zygotic division to create an individual that is a patchwork of the two genotypes. Elevated levels of PMS indicate a defect in MMR during meiotic recombination.

No other genes were known to serve such central roles in NER, MMR, and HR. When the *mei-9* gene was cloned and analyzed, it became clear that it was related to the NER incision enzymes Rad1p of yeast and XPF of humans *(155).* These proteins are part of a nuclease that cleaves one DNA strand 5′ of bulky lesions. Rad1p interacts with Rad10p, (as does XPF with ERCC1) to comprise a structure-specific endonuclease. The structure that is recognized and cleaved is the single-strand to double-strand transition at the 5′ end of the excision-repair bubble. A similar structure is presumed to occur in intermediates of reciprocal exchange in the form of Holliday junctions. Like several MMR genes, *RAD1* was recently discovered to play a role in regulating recom-

bination between closely related sequences containing heterologies *(33,122).* How this might relate to the role(s) of *mei-9* in meiotic crossing-over makes an engaging discussion *(154).*

3.3. MMR in Drosophila Extracts

In 1990, Holmes, Clark, and Modrich demonstrated a heteroduplex repairing activity in extracts of cultured Drosophila cells *(72).* For correction to be efficient, one stand of the substrate plasmid had to be nicked, and repair was heavily biased to the nicked strand. They found that G-T mismatches were more efficiently repaired than G-G or A-C. C-C mismatches were repaired less frequently than any of those above. This repair was associated with DNA synthesis in the region between the mismatch and the nick, consistent with the MMR model from *E. coli (72).*

More recently, Bhui-Kaur et al. *(10)* established that base:base mismatches can also be repaired very efficiently by extracts prepared from Drosophila embryos or adult cells. Of all the mismatches tested, only G-G, T-G, and to some degree C-C were shown to be repaired in a nick-dependent way. For these three mismatches, the efficiency of repair was higher when one strand was nicked, and the nicked strand was preferentially repaired. In contrast, repair of G-A, C-A, A-A, C-T, and T-T mismatches was not specific to the nicked strand. The authors suggested that G-A, C-A, and A-A, may be repaired predominantly by a mechanism involving an adenine-glycosylase rather than MMR. Similarly, the C-T, and T-T mismatches might be repaired by a thymine-glycosylase. Indeed, A-glycosylases and T-glycosylases have been found in other organisms *(10).* However, damaged bases rather than mismatches are thought to be the substrates of primary relevance for these repair enzymes. Recently it was found that mouse cells (and to a lesser degree human cells) devoid of long-patch MMR are able to efficiently and specifically repair A-C mismatches *(127).* In this case, only the A is replaced and no nick is required. These cells were also able to repair A-G mismatches with a low efficiency but no significant repair of other mismatches occurred. Drosophila has a gene similar to *8-oxoguanine DNA glycosylase (OGG1),* and a putative thymine glycosylase. Enigmatically this glycosylase matched *TDG,* a G:T specific thymine-DNA glycosylase that primarily repairs deaminated 5-methylcytosines by "base-excision repair." Until recently it was thought that there was no methylated DNA in *Drosophila.* It has now been shown that 5-methycystine is produced, but only during the early embryonic period *(102a).*

The nick-directed, long-patch MMR activity may be inducible by X-rays because specific activities of extracts were found to increase five to six-fold after X-irradiation *(10).* Also, the *mei-9* gene is required specifically for the nick-directed MMR in this *in vitro* system. Extracts from *mei-9* mutants were 5- to 12-fold less efficient at repairing G-G and T-G mismatches than those from wild-type flies, yet their activity on A-G, G-A, and C-C mismatches was not affected *(10).*

4. DOUBLE-STRAND BREAK REPAIR

4.1. Introduction

The DNA in living cells sustains spontaneous double-strand breaks (DSBs). Replication-fork disintegration is probably the most common route by which breaks arise, and through attempts to replicate damaged DNA *(31,143).* DNA breaks are also induced by

ionizing radiation and certain chemicals. In addition, there are special circumstances under which breaks are formed as part of a developmental program (e.g., meiotic recombination and V(D)J recombination). In order to study DSBR, it is very useful to be able to direct when and precisely where the breaks are made. Several methods have been developed to achieve this goal.

Traditionally, the repair of DSBs has been categorized into two classes: 1) HR, which requires that the broken end(s) pair with an intact homolog (or other homologous sequence) and use it as a template for DNA synthesis; and 2) nonhomologous end-joining (NHEJ), in which ends are rejoined without consulting a homologous sequence. It is now clear that even within these two categories multiple pathways are used to accomplish repair. Many DSBR studies have been performed in *S. cerevisiae,* but yeast apparently has a different relative preference for HR and NHEJ than higher eukaryotes. This is one of the reasons why studies of DSBR in *Drosophila* should be valuable.

4.2. Analysis of DSBR In Vivo: Germline Events

4.2.1. P Element DSBR System

For the last 10 years, the premier tool for analyzing repair of DSBs in Drosophila has been the transposon known as the P element. This tool was honed after the discovery that P elements move by a cut and paste mechanism. When they jump, they leave behind a DSB at the "donor" site. These breaks are usually repaired by HR, i.e., gene conversion (Fig. 1). The initial clue that implicated gene conversion was the discovery that a P element in the *white* gene underwent precise loss much more frequently when a homolog was present *(40).* Gloor et al. *(54)* proved that homologous sequences could direct the repair of the donor site by following the transfer of sequence polymorphisms from an engineered template to the site of P element excision. This demonstrated that the phenomenon could be used for targeted gene replacement as well as to study DSBR.

Examining the fate of such donor sites has yielded many insights into how DNA breaks are repaired. In the most prolific version of this system, P-induced breaks are created by crossing flies that contain a nonautonomous element (lacking transposase) to flies that contain a stable transposase source. Typically, the repair products are not analyzed in the flies that suffer the breaks, but in their progeny. This is desirable because the progeny are usually homogeneous and depict the result of a single repair event. Even though this strategy examines products that arise in the germ-line, it is known that the majority are from pre-meiotic repair because a pronounced clustering of conversion events is detected. Engineered-sequence polymorphisms also allowed properties of the conversion tracts to be scrutinized. The average length of these conversion tracts is about 1.4 kb. Most tracts are contiguous, i.e., not interrupted by unconverted sites, and most extend bi-directionally from the breaksite *(54).*

4.2.2. Template Preference

Conversion frequencies reveal a hierarchy of preference for template utilization. The order of preference is: sister chromatid > allelic site containing a P element at the identical position > allelic site (without P) > allelic site (without P) on a multiply inverted homolog > ectopic site in cis > ectopic site on a homolog > ectopic site on another chromosome. This order can be partially explained by proximity and by the extent of identity between the broken ends and the potential template. Of course sister chromatids are

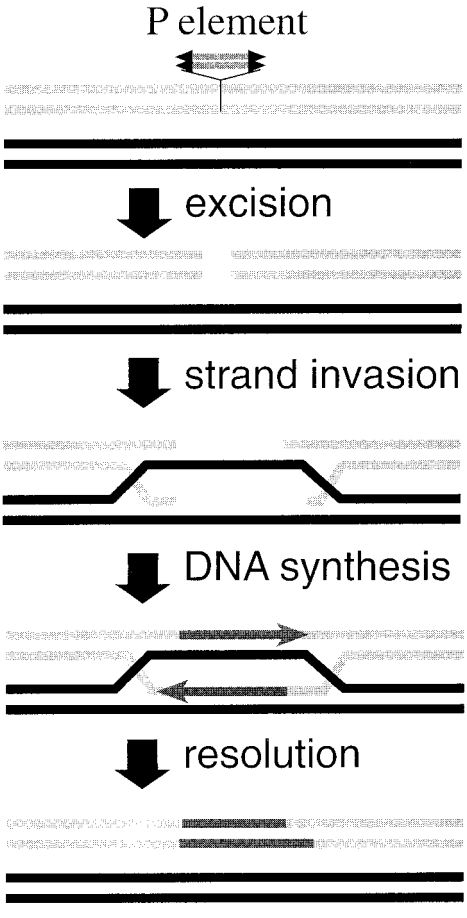


Fig. 1. P element-induced gene conversion. When a P element is mobilized, it leaves behind a DSB. Frequently the broken chromosome is repaired by gene conversion, which can be guided by the corresponding sequence from its homolog. In this depiction, two Holliday junctions are formed and then resolved to yield noncrossover products.

identical before the P element excises, whereas a homolog, though very similar, will often contain polymorphisms in the vicinity of the break. If the entire P element sequence is excised during transposition, it should not matter whether or not a potential template carries a P element at the same site. However, cleavage by P transposase generates 17-base, 3′ overhangs that consist of the terminal 17 bases of the element *(9)*. Junctions with these 17 bases only match a homolog with a P element at the same site (Fig. 2). Indeed, the presence of 17 bases of terminal heterology has a major effect. Compared to a homologous chromosome without a P element, homologs that have a small P element fragment containing at least the 17 bases from both termini, greatly increased conversion *(77)*. The quality of the sequence match is also very important. In one study using homologous chromosomes as templates, the number of single bp heterologies was varied within a ~3.5 kbp region. The frequency of gene conversion dropped from 19% when no mismatches were present to only 5% when the template had 15 mismatches *(119)*.

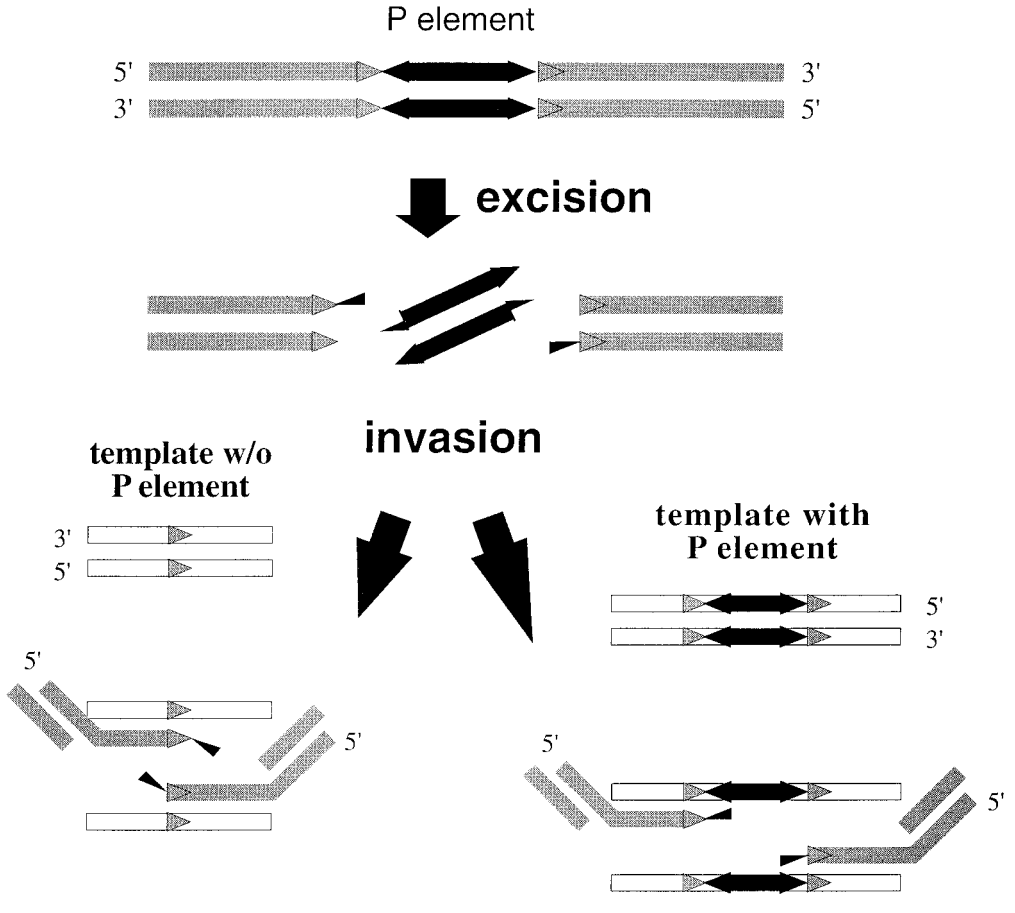


Fig. 2. P element excision produces unusual breaks with 3′ overhangs of 17 bases. Repair of the "donor" chromosome is influenced by the presence of 17 bases of P element sequence. Templates that contain a P element at the same site support a much higher frequency of conversion than templates without P. Left, The 17 base extensions must be removed before repair synthesis can occur on the template lacking a P element. Right, An invading 3′ end can anneal perfectly to the P-containing template and is ready to be extended by repair synthesis.

Another plausible component of the template hierarchy might be the average physical distance between template and breaksite. Sites on sister chromatids could be expected to be close, especially soon after replication. Homologs may be partially paired throughout the cell cycle, but multiple inversions may disrupt pairing *(47,55,70).* It is unclear how a template located several Mbp distant can be recognized as co-linear and utilized preferentially, considering how the nucleus often resembles a mass of spaghetti *(41).* A linear-tracking model seems untenable but alternative models, such as one where each chromosome occupies a compact nuclear domain, seem equally unfeasible.

Extrachromosomal circular plasmids can also be used as templates for P-induced conversion. For multiple reasons it is difficult to compare the efficiency of plasmid and chromosomal templates. Plasmids are injected into the embryos at a many-fold excess to the number of chromosome copies. Also plasmids persist for a fraction of the fly's lifespan, and the copy number probably varies greatly from cell to cell. Still the fre-

quency is high enough that plasmids can be used for gene targeting and transformation of Drosophila by DSBR *(81)*.

4.2.3. Homology Requirements

Gene-targeting studies demonstrated that both insertions and deletions can be efficiently copied into a P element-induced breaksite if they are encompassed within homologous sequence. As mentioned earlier, mismatches and terminal homology affect template efficiency during gene conversion *(77,119)*. Although different types of heterologies affect the overall rate of conversion differently, among conversion events the frequency of inserting 8 kbp, deleting 136 bp, or creating a single-base change is the same *(120)*. Several other experiments have addressed the minimum amount of homologous sequence required for efficient conversion. One study used a break at w^{hd} (a P element insertion in the eye-color gene known as *white*) and a set of ectopic templates containing ~3 kbp of homology to the left of the breakpoint and varying amounts to the right. On the right of the breakpoint all templates began with 238 bp of matching sequence followed by an 8 kb heterologous insertion then resumed with either 0, 25, 51, 375, or 493 bp of additional matching sequence. The rate of conversion of the heterologous insertion was very low when the outer homology was 51 bp or less. Conversion rates were moderate with 375 bp of homology and high when 493 bp of homology was present *(35)*.

In most experiments that use ectopic templates, an interesting class of aberrant events is recovered. These peculiar conversions all retain one P element end at a position that corresponds to a P terminus in the donor. They also contain a sequence duplication beginning at that same point. These aberrant products, dubbed "conversion duplications," can be explained if the terminal 17 bp are sufficient to direct recombination at one end *(120)*. The 17-base tail may either prime synthesis from a P end in the template or serve as the site of alignment when the two sides of the break come together after template-directed synthesis (Fig. 3). The view that 17 bases is sufficient is also supported by the transposon swap phenomenon *(48,58,69,82,107,156)*. In a stock that has two distinguishable P elements, one can precisely replace the other when mobilized. Presumably, gene conversion allows the 17-base tails to find the ends of the other P element anywhere in the genome and copy the sequence of that element into the break. It is a formal possibility that more than 17 bases are required for these events if they occur in two steps. In that case, initial repair of the break could use the sister chromatid to extend the P element sequence to 31 bases or more (note that the terminal 31 bases of P elements are perfect inverted repeats). The extended tails could use the additional homology to pair with the second P element. However, Preston and Engels succeeded in converting a DS oligo into a P-induced break guided only by the 17 bases of P on both sides *(137)*. Current data suggest that although 17 bases of matching sequence is too short to produce the highest conversion frequency, it can suffice.

4.2.4. Synthesis-Dependent Strand Annealing

Conversion duplication products were instrumental in the development of a new model of HR called synthesis-dependent strand annealing (SDSA) *(120)*. In SDSA, after a DSB is formed, the two 3′ ends independently invade and copy homologous template(s). This creates long single-stranded tails that anneal at complementary regions. The annealed strands are processed until fully double-stranded by any necessary synthesis and/or trimming and repair is completed by ligation (Fig. 4). The SDSA model is distinct in that it does not

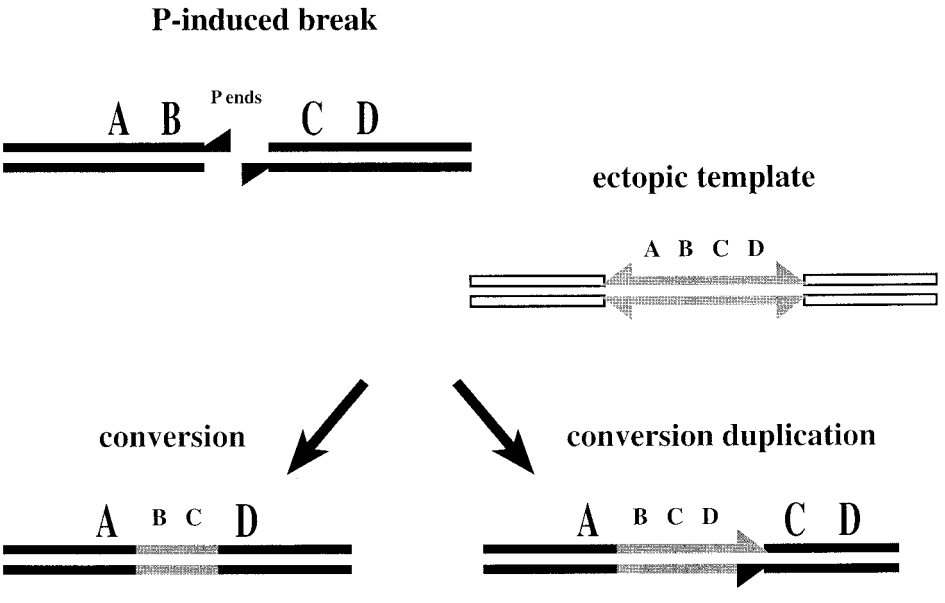


Fig. 3. An example of a "conversion-duplication" structure. When P-induced DSBR utilizes a template that resides ectopically within another P element, a few percent of the events recovered are of the "conversion-duplication" class. The structure suggests that alignment on one side of the break has been directed by the termini of the two transposons.

invoke Holliday junctions but rather migrating repair "bubbles," and it entails the association of long single-strands as in single-strand annealing *(166).* These features can easily explain the origin of the conversion duplications and are in accord with the fact that conversion is only infrequently associated with crossing-over. In contrast, models that include resolution of Holliday junctions predict frequent crossing-over. SDSA may be a widely used mechanism because this model best explains results from a variety of settings including alterations in sequence repeats in yeast and rye, intron homing in bacteriophage T4, and formation of defective transposons in Drosophila and maize *(11,91,117,120,133,145).* The strongest evidence for SDSA comes from recombination products that include sequence information from two separate templates *(76,133,135).* No other model can easily explain such "bitemplate" events.

4.2.5. Effects of Heterologous Insertions

Despite the fact that large insertions are included within successful conversion tracts as frequently as single-base differences, they do not affect conversion in the same way. The amount of flanking homology required is greater for large heterologies *(36).* Also the overall frequency of conversion is strongly affected by large heterologies (~8 kb) located 238 bp from the breaksite *(35).* The frequency is less affected by shorter heterologies (242 bp to 4 kbp) and unaffected by short (25 bp) insertions *(35).* It seems that the proximity to the break is a crucial factor, because a large insertion 2 kbp from a breaksite had little effect on conversion frequency *(40).*

4.2.6. Possible Chromatin Effects

The fact that homologous templates can be found at all is astonishing when the true state of chromosomal DNA is considered. The DNA in cells is compacted by several

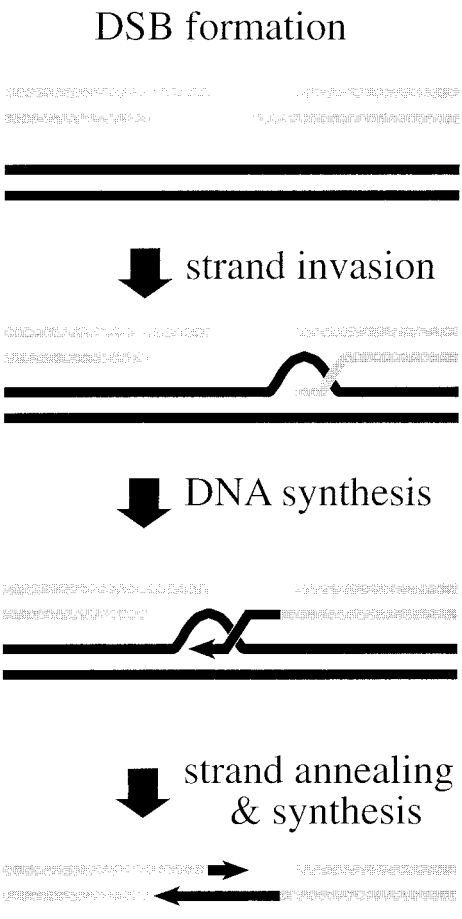


Fig. 4. Synthesis-dependent strand annealing. *See* text for details. For simplicity, this depiction shows invasion and synthesis initiating only with the broken right end. Apparently both ends can invade and extend independently, sometimes involving separate homologous templates.

levels of organization into chromatin. Evidence is mounting that chromatin remodeling is a very important aspect of nuclear activity that is capable of regulating processes such as transcription and DNA repair *(115).* The absence of one chromatin "insulator" protein known as *suppressor of Hairy wing (su(Hw)),* leads to an approx fourfold increase in conversion rates when an ectopic template is used *(93).*

4.2.7. Other DSB Systems

Other methods have been used to make DSBs in Drosophila chromosomes and these have been used to test which aspects are general to DSBR, and which are specifically influenced by P element idiosyncrasies. Repair of chromosome breaks caused by the rare-cutting endonucleases HO and I-*Sce*I have been analyzed *(78,137).* So far, the general conclusion is that most of the characteristics of P-induced DSBR are shared with these other systems. For example, when the recognition site for HO endonuclease is placed in the *white* gene on the X chromosome, conversion-tract distributions are remarkably similar to those recovered by P mobilization *(78).* When conversion uses the homologous X chromosome as a template, there is a similar, low level of associated

crossing-over. Ectopic templates located in *cis* to the break are used preferentially just as is the case with P elements. Also, examples of repair utilizing two different templates, so-called bi-template events, have been recovered, suggesting that both ends produced by HO cleavage can independently invade templates, again similar to the case with P elements *(78)*.

I-*SceI* endonuclease cuts Drosophila chromosomes very efficiently, but the relative frequency of the alternative outcomes (end-joining, conversion with or without crossing-over) is still under investigation *(137)*. Conversions can be recovered at a rate comparable to P-induced DSBR and again, as with P, crossovers are much rarer than conversions *(137)*. When I-*SceI* cuts are made between direct repeats of homologous sequence, which provides an opportunity for single-strand annealing, very high rates of recombination are seen *(141)*.

Another transposon known as the mariner element also induces homolog-directed gene conversion when it excises in Drosophila *(101)*. Mariner is a member of the extremely widespread mariner/Tc1 transposon superfamily—the genomes of many plant and animal species are replete with these elements. Faulty DSB repair after mariner excision also appears to be the source of defective copies of mariner *(101)*. Similar to the scenario with P elements, aborted or inaccurate gene conversion replaces an internally deleted element into the site of the excised transposon.

4.2.8. Gene Targeting

Until recently, targeted-gene replacement in Drosophila was limited to the immediate vicinity of P element insertions or other engineered sites of DSB formation. The first successful targeted disruption of an unmodified gene was reported by Rong and Golic *(141)*. In most targeting systems, DNA ends are required for high levels of recombination, but all attempts to provoke gene replacement by injecting linear substrates into fly embryos have failed. The trick seems to be to create linear, extrachromosomal recombination donors in vivo *(141)*. A donor cassette was engineered and inserted randomly into the Drosophila genome by P element mediated transformation. The cassette contained a segment of the targeted gene with an I-*SceI* cleavage site in the middle. This was flanked by two FRT sites, (substrates for FLP recombinase), arranged as direct repeats. The action of FLP recombinase on these sites loops out the intervening sequence creating an extrachromosomal circle and at the same time reseals the chromosome that is now deleted for this sequence. Cleavage by I-*SceI* linearizes the circle creating the (presumed) active donor with recombinogenic ends.

Recombinant products were recovered by selecting for homology-directed reversion of a mutation at the target locus. The homologous sequences at the ends were oriented to form an "ends-in" donor, so the expected outcome was integration at the targeted site producing a tandem duplication *(63)*. About one-third of the selected products had the expected configuration *(141)*.

The other two-thirds of the products were composed of three structural classes that can be explained by a mechanism in which dimers of the circular donor arise prior to integration. Indeed, about 7% of the products appeared to be simple integrations of a dimerized donor, thus creating tandem triplications *(141)*. This arrangement should be unstable if FLP recombinase or I-*Sce*I is still present. The central copy of the triplication would be efficiently excised by FLP because it is flanked by FRT sites (excision would produce a configuration identical to the simple "ends-in" integration, therefore some in the expected

class could originate through donor dimers). The central copy of the triplication would contain an I-*Sce*I cleavage site, so if any I-*Sce*I endonuclease remains, it would provoke a second round of DSBR. One of the likely products of such secondary repair would be simple "allelic replacement" of the targeted mutant allele with the donor allele. This outcome could ensue from a cleaved triplication by gene conversion, single-strand annealing, or sister chromatid exchange. Fully one third of the recombinants recovered were "allelic substitution" products, yet they are not easily explained without assuming donor dimerization *(141).* DSBR of the triplication is also likely to produce some events that collapse the structure to duplications identical to the simple integration products. The final class (23% of the total) has a structure similar to the expected tandem arrangement except that one copy of the duplication has a small deletion or insertion near the position of the I-*Sce*I site *(141).* These scars are reminiscent of NHEJ, yet homology undoubtedly directed integration. A donor dimer could suffer inactivation of one of its I-*Sce*I sites by NHEJ and still be targeted through cleavage at the other site. Integration after extensive degradation of one end by exonuclease could result in the observed structures. But how could dimers form when there is only one copy of the cassette in the genome in the first place? The simplest theory is that dimers arise through replication. If the circles could replicate, multimers would be formed readily by FLP mediated co-integration or I-*Sce*I mediated DSBR. Alternatively dimers could arise (intra- or extrachromosomally) in G2 cells by interaction of copies from the sister chromatids. However, it is unclear why dimers appear to be the favored substrates. What key recombinational advantage might dimers provide? Because of the excitement over this new gene-targeting scheme, details of the mechanism (whether dimers are involved or not) should emerge quickly.

4.3 Analysis of Somatic DSBR

There is evidence that the relative efficiency of gene conversion and NHEJ is different in Drosophila somatic cells vs premeiotic germ cells. Also the size of the most frequent deletions created by NHEJ in somatic cells seems to differ from those arising in premeiotic germ cells *(8,44,53,77,78,125,161,169).* To a large degree the apparent disparity can be explained by differences in experimental designs. Often a phenotypic screen was used to select the product before analysis. In some experiments, a large number of copies of linearized plasmids were injected into embryos *(8,125),* whereas in others, repair of a single break at a chromosomal site was studied *(44,53,77,78,161,169).* However, even when similar conditions are used, there are persistent differences in somatic and germline repair *(53).* Unfortunately it is not possible to control for variability that results from the different life histories of diverse tissues. Factors such as the number of cell divisions, rate of DNA synthesis, and duration of cell-cycle phases may affect the ratio of the types of products recovered and obscure whether certain repair pathways are truly modulated in somatic verses germline cells. There is clear evidence of cell-type-specific regulation of DNA repair in yeast (*see* Chapter 5).

4.4. Analysis of DSBR in Embryos

4.4.1. P Element Loss, Reversion Assays, and NHEJ

Assays for DNA repair after transposon excision have been performed in Drosophila embryos *(126).* Plasmids bearing a transposon were injected into preblastoderm embryos. Transposase was produced either from a co-injected plasmid or a chromosomal source. In most of these assays, repair was analyzed phenotypically after plasmids

were re-isolated from embryos and transformed into *E. coli.* This type of assay selects for a small subset of repair events that restore the function of a bacterial gene that the transposon has disrupted. Because there is no opportunity for homologous repair, NHEJ events that produce small in-frame insertions or deletions are recovered. Despite the severe bias inherent in such assays, they have been very useful. For example, it was shown that γ-irradiation can enhance the recovery of these repair products *(61),* whereas mutations in *mus309* diminish recovery *(8).*

4.4.2. Homologous Recombination (HR) and Nonhomologous End-Joining in Embryos

The repair of plasmids that have been linearized in vitro can also be assayed in Drosophila embryos *(37,59).* Linear DNA substrates are injected into the early embryo prior to cellularization. Intramolecular repair and some HR events lead to plasmid recircularization, which can be followed by PCR or analyzed after transformation of *E. coli.*

One study of DSB repair in embryos analyzed the sequence of 122 NHEJ junctions *(37).* Most of these, (117 events) had deletions of plasmid sequence, but a quarter of the junctions had lost nucleotides from only one side of the break. Slightly more than half of the deletions were <10 bp, 41% were 10–100 bp, and 7% longer than 100 bp. The addition of bases that are not present in the original plasmid was also a common feature, but most of the insertions were less than 20 bases. As seen in other studies *(88,134,142,144,149),* almost all of the products that did not have insertions of extra bases had been joined at regions of microhomology. These junctions contained one to three bases that could have come from either side, suggesting that repair was influenced by short regions of complementarity *(37).*

Intermolecular HR could be detected when a homologous fragment was co-injected with the plasmid. The homologous fragment was designed to span the site of plasmid cleavage and contained 18 additional basepairs at the exact position of the break. Thus homologous repair using the fragment as a template is revealed by transfer of these 18 bp into the plasmid. This type of repair product was readily detected by PCR even though NHEJ appears to predominate. Irradiation of the embryos with γ-rays prior to substrate injection was found to stimulate HR, especially the production of mature recombinant circles capable of transforming *E. coli (37).*

Another study used embryos in a similar way to investigate both intramolecular and intermolecular HR as well as NHEJ *(59).* The substrate for intramolecular HR and NHEJ was a linearized plasmid that contained two copies of a short sequence. HR within the duplication would recircularize the plasmid and reconstruct a tetracycline resistance gene. NHEJ was much more frequent than intramolecular HR. As in other studies, deletions were common at these NHEJ junctions. The average length of deletion was 14 bp. Intermolecular HR was also assayed with a linearized plasmid and a homologous fragment. In this case, recombination was detected by (homology-directed) reversion of a mutation near the site of plasmid cleavage. Once again, HR was easily detected although it was not very efficient compared to NHEJ *(59).*

4.5. Genes Involved in DSBR

4.5.1. spindle Genes

Recently the studies of oocyte development and DSBR converged with the surprising discovery that several mutants defective in embryonic axis formation had lost DSBR functions. These so-called spindle-class genes were isolated by selecting mutations that

produced ventralized embryos lacking dorsal structure. Such embryos are narrower, more pointy, and more symmetrical than wild-type (hence the name spindle). This phenotype is caused by the inability to establish dorsal/ventral polarity that is the direct result of a failure to accumulate GURKEN protein in the anterior-dorsal region of the oocyte *(49,57).* Remarkably, three of the spindle-class genes that have been identified appear to have a primary role in DSBR/meiotic recombination. These include a *RAD54* homolog, a *RAD51/DMC1* homolog, and a third DNA repair gene, *mus301.*

How do mutations in repair genes lead to developmental defects of the oocyte? These mutants cannot complete repair of meiotically induced DSBs that initiate crossing-over. This block in DSBR activates a meiotic checkpoint so that the oocytes do not complete prophase I. As part of the response to this checkpoint, VASA protein is modified, which in turn inhibits *gurken* mRNA translation. Without sufficient GURKEN, the dorsal-ventral axis cannot be formed. In a *mei-W68* mutant background, no meiotic breaks are made, and the DSBR-deficient spindle class mutants are able to progress through prophase I. Alternatively, if the oocyte has a faulty meiotic checkpoint, as in a *mei-41* mutant, the DSBR-deficient spindle class mutants are again able to proceed through prophase I *(50)* (Fig. 5).

4.5.1.1. *SPN-B*

The SPN-B protein is a member of the RecA/RAD51 family *(49).* Several proteins in this family have been shown to catalyze strand-transfer in vitro, to play roles in DSBR, and to form specific complexes during meiotic recombination. The *spn-B* message is expressed throughout oogenesis. It has not been reported whether it is expressed in other stages and tissues *(49).* Mutations in *spn-B* are not known to affect mitotic DNA repair. For example, they are not hypersensitive to MMS *(49),* but they do have several defects in meiotic recombination. *spn-B* mutant females are nearly sterile. In the rare offspring, meiotic crossovers are decreased 4- to 10-fold and X-chromosome nondisjunction (NDJ) is increased about 100-fold. The oocyte chromosomes of *spn-B* mutants often fail to form into a compact karyosome but remain diffuse and thread-like *(57).*

Among RAD51 family members, SPN-B is most closely related to human XRCC3 and RAD51C (about 35% identical, 49% similar amino acids). It also shares about 27% identity/43% similarity to human DMC1 and 30% identity/44% similarity to human RAD51. Owing to its conspicuous meiotic phenotypes, it has been compared to DMC1, which is exclusively expressed during meiosis in yeast and mammals and is indispensable for meiotic recombination. It is possible that the full spectrum of *spn-B* function has not been uncovered yet.

4.5.1.2. *SPN-D*

The molecular identity of *spn-D* has not been revealed, but it produces a mutant phenotype very similar to that of *spn-B.*

4.5.1.3. *OKR (DMRAD54)*

Another spindle-class gene was named *okra (okr).* Female *okr* mutants, are also sterile, producing ventralized embryos. Unlike *spn-B* and *spn-D* mutants, *okr* mutants are hypersensitive to MMS. The *okr* gene encodes the Drosophila homolog of *RAD54.* It shares 54% identical amino acids with human RAD54 and 48% with yeast Rad54p *(49,87).* Hypersensitivity to MMS is also seen in yeast *rad54* mutants. Yeast Rad54p

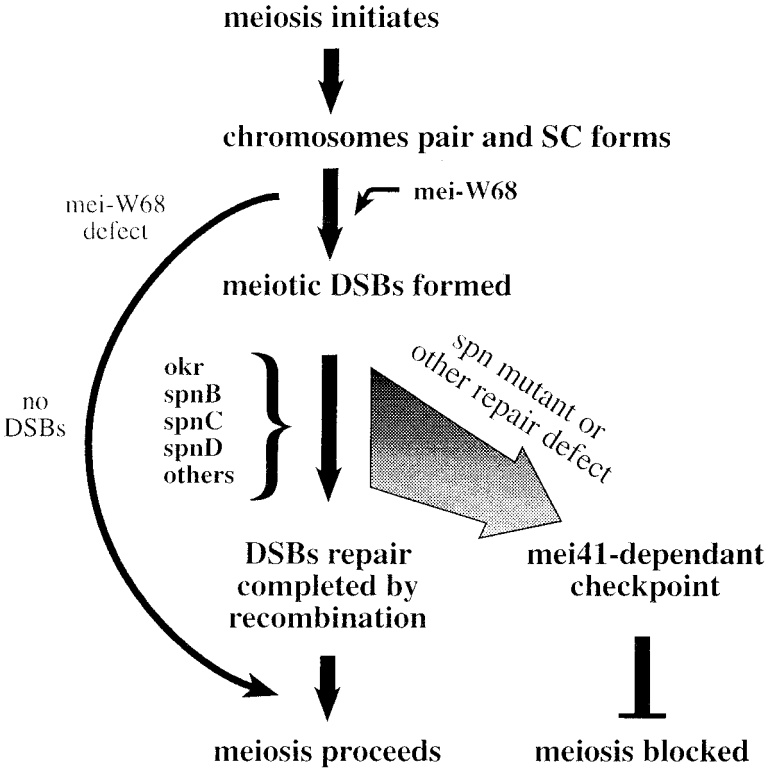


Fig. 5. A DSB-induced meiotic checkpoint. MEI-W68 appears to be required for the formation of meiotic DSBs while some of the spindle-class genes are directly involved in the repair of these meiotic breaks. If the breaks fail to be repaired, as in a *spn* mutant, a *mei41* -dependant checkpoint arrests the progress of meiosis. Oocytes mutant for *mei-W68* and a *spn* gene circumvent this checkpoint because no DSBs are produced.

participates in DSBR and HR. It can stimulate RAD51p-directed homologous pairing, and appears to have a particularly important role in DSBR utilizing the sister chromatid. Consistent with a DSBR function, both yeast and Drosophila *rad54/okr* mutants are hypersensitive to X-rays *(87).* In addition, flies with *okr* mutations are hypersensitive to breaks made by P element excision and to the crosslinking agents, mitomycin C (MMC) and cisplatin *(86).* Mutations in *okr* and *mus309* (Subheading 4.5.1.4) are synergistic with respect to X-ray sensitivity, but roughly additive with respect to MMS sensitivity *(86).*

In an assay for induced loss of heterozygosity and HR in somatic cells, *okr* mutations had little affect when the inducing agent was MMS, MMC, or cisplatin. In contrast, *okr* mutations abolished the recovery of X-ray-induced loss of heterozygosity events *(86).* Transcription of *okr* occurs at all developmental stages but is highest in ovaries and early embryos *(87).* Yeast and mice mutant for *rad54* have little or no defect in meiosis. Strong *okr* mutants, on the contrary, are completely sterile in females, apparently unable to repair meiotic DSBs. Meiotic recombination and NDJ have been measured in weak (nonsterile) *okr* mutants. These mutants produce about 50% of the normal level of recombinants and about 20-fold more NDJ events *(49).*

4.5.1.4 *spn-C (mus301)*

Mutant *spindle-C* flies also lay ventralized embryos and are sensitive to MMS. The *spn-C* gene is allelic to the mutagen sensitive gene, *mus301*. A mutant P element-insertion line failed to complement both *mus301* and *spn-C* and *spn-C* mutants do not complement mutant *mus301* alleles for MMS sensitivity *(50,152).* There are five mutant alleles of *mus301* and these yield varying degrees of sterility when homozygous. As in *spn-B* and *okr,* females with partially fertile alleles of *mus301* produce increased frequency of NDJ *(16).* Hypersensitivity to X-rays is also seen in *mus301* mutants *(129).* Intriguingly, the *mus301* gene maps to a region that contains a homolog of *recQ4 (89)* (*see* Subheading 4.5.6.2.).

4.5.2. mei-41(ATR, MEC1)

MEI-41 is a member of a protein family whose C-termini are related to phosphatidylinositol 3-kinases *(62).* Many members of this family are DNA damage-inducible checkpoint signaling proteins including yeast Mec1p and human ATM and ATR. The *mei-41* gene derives its name from the defect in meiotic recombination that results from "weakly" mutated alleles *(27).* Such alleles produce reduced levels of meiotic exchange with concomitant increase in NDJ. Strong mutations cause female sterility through maternal-effect embryonic lethality *(159).* Mutations also cause severe sensitivity to DNA damaging agents *(17,105)* and P element-induced breaks *(5).* MEI-41 has an essential role in embryonic development in a unique DNA replication/DNA damage checkpoint that is necessary for the transition from the extremely rapid, maternally programmed, early cell divisions to the zygotically controlled divisions after the midblastula stage *(159).* The Drosophila *CHK1* homolog, *grapes,* and Drosophila *wee1* are also components of this pathway *(138,159).*

MEI-41 has another intriguing role in female meiosis. In wild-type oocytes the presence of chiasmata leads to arrest in metaphase I. Certain mutants defective in meiotic crossovers but not repair by gene conversion (i.e. *mei-9* and *mei-218*) show early entry into anaphase. This precocious entry into anaphase requires MEI-41 *(113).* One possibility is that MEI-41 may be required to extinguish a signal that normally arrests meiotic progression until DSBR is complete *(154).*

4.5.3. Ku70

Mammalian cells possess several protein complexes that sense the presence of DSBs and alert the cell to elicit the appropriate response. One such complex is DNA-dependent protein kinase (DNA-PK). DNA-PK is made up of a large subunit that encodes the protein kinase activity and a heterodimer of two smaller subunits, Ku80 (or Ku86) and Ku70, that constitute a DNA end-binding activity. The Ku86/Ku70 dimer must be bound to DNA in order to stimulate the catalytic subunit of DNA-PK. Activated DNA-PK phosphorylates several DNA binding proteins including p53 and is required for p53-mediated apoptosis. Besides its role in signaling and cell-cycle checkpoints, the Ku heterodimer has been implicated in the repair process itself. Evidence suggests that Ku protects DNA ends from excessive nucleolytic degradation and may in fact tether the two ends together to facilitate repair *(13,14,29,97,131).* Ku is also involved in protecting and maintaining telomeres.

Drosophila Ku70 was originally isolated during a search for inverted repeat binding protein (Irbp), a factor that binds to the outer half of P element terminal repeats *(7).* The bound proteins were purified and antibodies were raised against peptide fragments.

This led to the isolation of a *Ku70* cDNA. Although they are likely to be a true homologs, the human and Drosophila Ku70s have diverged significantly, retaining only 27% identical amino acids. Drosophila *Ku70* was independently cloned as yolk protein factor 1b, *(Ypbf1b)* by virtue of its apparent sequence-specific DNA-binding within the *Yp1* gene *(74).* When the *Ku70* gene was mapped cytogenetically, it was found to reside in the vicinity of a gene called *mus309.* Beall and Rio *(8)* showed that a genomic fragment containing the *Ku70* gene partially rescued female sterility and MMS sensitivity of *mus309* mutants. Therefore, *Ku70* and *mus309* were considered the same gene despite discrepancies in map positions and predicted phenotypes. A gene encoding Ku86 was recently identified *(51).*

4.5.4. mus309 (blm)

Several of the phenotypes of *mus309* mutants could conceivably be attributed to defects in DSBR including reduced fertility, reduced viability (especially in males), altered frequency of meiotic recombination, increased NDJ and chromosome loss, and increased mitotic exchange. In addition, more specific studies have shown that mutations in *mus309* affect the repair of DSBs induced by P element transposition, HO endonuclease, and X-rays *(78,86).*

The *mus309* gene was identified in 1981 *(16).* Three mutations, *mus309*^{D1}, *mus309*^{D2}, and *mus309*^{D3}, were determined to be allelic because they failed to complement each other for recessive sensitivity to MMS and nitrogen mustard. However, the D1 allele was recovered with a linked recessive lethal, and the D3 allele was associated with a second (unidentified) mutagen-sensitive mutation. The D2 allele has a recessive female-sterile phenotype. The heteroallelic combination *mus309*^{D1}/*mus309*^{D2} causes sterility in both females and males *(16),* and both *mus309*^{D2} and *mus309*^{D3} are female sterile over a deletion *(167).* The D1 allele has been lost and thus, most analyses have been performed with a combination of the D2 and D3 alleles even though these flies are only weakly fertile.

Preliminary results from *mus309*^{D2}/*mus309*^{D3} mothers show that their sons survive ~five-fold less often than daughters. This sex bias is absent in the progeny of *mus309* mutant fathers *(78).* About 4% of the total progeny, (over 25% of the males) from *mus309* mothers are XO (null Y). These flies could derive from maternal chromosome loss or meiotic NDJ. The frequency of XO males is at least 75-fold higher than in wild-type controls. The occurrence of XXY females, which is indicative of NDJ, is elevated to a lesser degree (~10-fold), implying that chromosome loss is contributing the major effect. Paternal chromosome loss and NDJ are also elevated in *mus309* mutant fathers *(78,89).*

mus309 mutations also affect meiotic recombination, but in a complex way. The recombination rate on the 2nd chromosome is reduced over-all by two-fold, but the effect is not uniform across the chromosome length. Exchange in the left and right arms is reduced two- to three-fold and increased ~three-fold in the central region *(78).*

As mentioned previously, there is no meiotic recombination in male Drosophila, so any recombinant chromosomes arising in the male germ line derive from nonmeiotic events. Therefore the rate of spontaneous recombination in the male germline is very low in a wild-type background, (less than 10^{-5} per chromosome per generation). In a *mus309* mutant the rate is increased over 2000-fold *(78).* Two possible explanations for this increase are (1) more DNA damage occurs (or persists) in *mus309* mutants, which leads to a higher recombination rate, or (2) the same amount of DNA damage occurs

but it more often results in crossing-over. Given the evidence for increased chromosome loss, the first scenario seems more likely.

Beall and Rio *(8)* showed that *mus309*^{D2}/*mus309*^{D3} flies are hypersensitive to P mobilization. The sensitivity was more severe in males, (~6-fold) than in females (~1.8-fold). This sensitivity is presumed to be caused by inefficient repair of DSBs. Because the mobilized P elements were inserted in the sex-specific X chromosome, the higher sensitivity in males may be owing to the absence of a homolog to serve as a repair template. Alternatively, the sex bias might be explained if recessive lethals are produced in both male and female *mus309* mutants but they are complemented by the homolog in females.

When P elements are mobilized from the X chromosome in the germline of wild-type males, deletions are found near the break site in about 30% of the offspring. In a *mus309* mutant, the proportion jumps to about 90% *(78).* In this assay, only one X homolog is present, so repair by HR can only take place when the sister chromatid is available. Nonetheless, one subclass representing 22% of the progeny have deletions within the body of the P element, indicating that repair synthesis using the sister as a template was initiated but failed to complete or resolve properly. This type of event is observed five times more often in the descendants of *mus309* fathers than those from wild-type fathers. This suggests that *mus309* is involved in HR.

In a similar assay, breaks were induced on the X chromosome in males that had an ectopic template for homologous repair. In this experiment, P element mobilization led to a "reversion" rate that was 1.5- to 3-fold lower in *mus309* mutants than wild-type controls. These "reversion" events represent successful repair from the ectopic template. In contrast, the rate of "marker loss," which is caused by internal deletions and flanking deletions, increased 2.5- to 5-fold in *mus309* mutants. Very similar results were obtained when the DNA breaks were produced by HO endonuclease cleavage rather than P transposition *(78).*

The idea that *mus309* mutants are defective in DSBR is supported by the fact that *mus309*^{D2}/*mus309*^{D3} mutants are three-fold more sensitive to X-rays than wild-type flies *(87).* A synergistic effect of *mus309* with *DmRad54* was also observed. Whereas *DmRad54*$^{-/-}$ mutants are seven-fold more sensitive, the *mus309*$^{-/-}$ *DmRad54*$^{-/-}$ double mutants are 40-fold more sensitive to X-rays than wild-type. The same double mutant combination does not show synergy in MMS sensitivity *(86).*

The *mus309* gene was recently shown to be the *Drosophila* homolog of the human Bloom syndrome gene, BLM. Deletion mapping, rescue by transgene, and the sequence of mutants all confirm that *mus309* is *Dmblm* rather thatn *Ku70 (see Subheading 4.5.6.1.; 89).*

4.5.5. mus209 (PCNA)

The *mus209* gene encodes the proliferating cell nuclear antigen (PCNA) homolog of Drosophila *(66).* The existing mutations cause recessive lethality or recessive temperature sensitive (ts) lethality. PCNA is involved in DNA replication, DNA repair, recombination, and cell-cycle regulation. It is a component of both DNA polymerase δ and polymerase ε. FEN-1 and p21$^{WAF1/Cip1}$ (called DACAPO in Drosophila), bind to the same site on PCNA. FEN-1 is also an essential replication factor; it is an endo/exonuclease required for processing Okazaki fragments during lagging strand DNA synthesis. Binding of p21 to PCNA is thought to inhibit replication by displacing FEN-1 *(173).* The ts-alleles of *mus309* cause recessive sensitivity to MMS, ionizing radiation, and

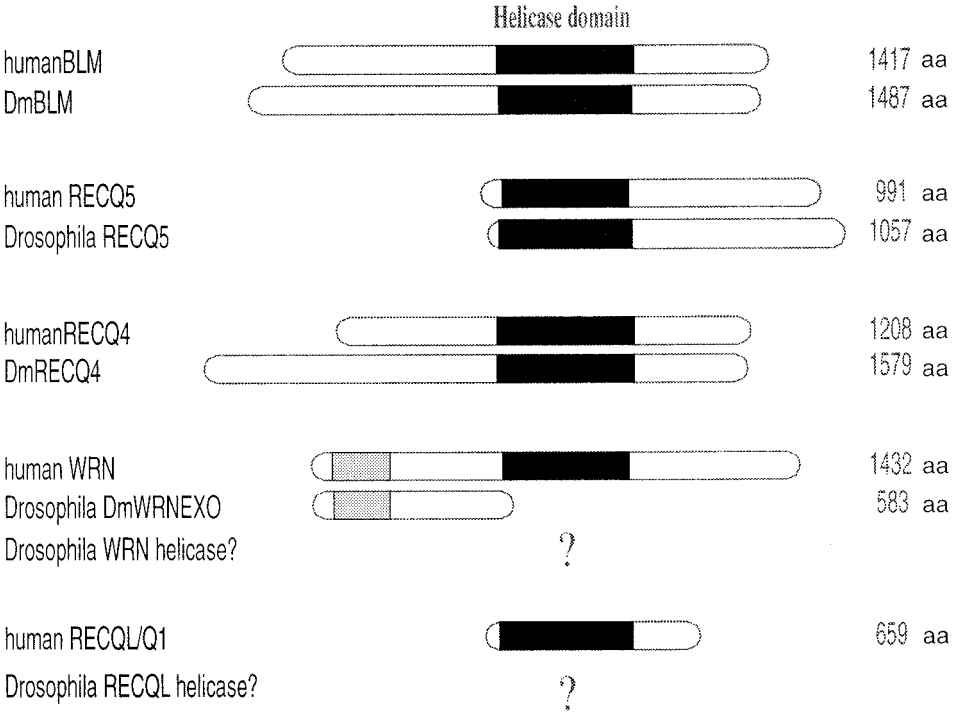


Fig. 6. Drosophila and human RECQs. The overall structure of the known RECQ proteins of Drosophila and human are compared. The locations of the conserved helicase domains are shown in black. The total number of amino acids predicted for each protein is indicated (note that RECQ5 and others may produce more than one protein by alternative splicing). A putative exonuclease, related to the WRN exonuclease domain, occurs in Drosophila within a smaller ORF that does not encode a helicase. Genes closely related to the WRN helicase and human RECQL have not been found in Drosophila.

bleomycin at the permissive temperature *(65–67).* PCNA is also required to repair P element-induced DSBs *(67).* Partial complementation of several of the mutant defects can be achieved with a combination of heteroalleles *(68).*

4.5.6. The RECQ family

The RECQ family is composed of a group of related proteins that contain the seven canonical helicase motifs and share sequence similarity both within and around these motifs. Several members have been confirmed as DNA helicases, being able to unwind the two strands of the double helix. RECQ members have roles in repair of DNA by HR and in genome stabilization *(30,79).* The RECQ family consists of at least four subfamilies. Eukaryotes frequently retain four or more *recQ* homologs, often including representatives from three of the subfamilies. This suggests that each group provides distinct functions *(90).*

4.5.6.1. *DmBlm*

Drosophila *Dmblm* gene is a RecQ family member that is closely related to the human *BLM* gene *(90)* (Fig. 6). Mutations in *BLM* cause Bloom syndrome, an autosomal recessive disorder characterized by growth deficiency, immunodeficiency, cancer

susceptibility, and chromosome instability. The DMBLM protein is also similar to *S. cerevisiae* Sgs1p, although it is more distantly related to Sgs1p than it is to BLM. Despite this divergence, *Dmblm* can partially rescue MMS hypersensitivity of *sgs1* yeast *(90),* indicating that functional similarities persist.

4.5.6.2. *recQ4*

Another RECQ homolog of *D. melanogaster,* DMRECQ4, is very similar to human RECQ4 within and around the helicase domain. Mutations in human RECQ4 cause Rothmund-Thomson syndrome *(84).* This syndrome manifests in abnormalities of the skin and skeleton, signs of premature aging, chromosome instability, and a predisposition to cancer *(83,99,172).* The *DmrecQ4* transcript is about 4.9 kb. The gene maps to 66B10-66C1 close to *spn-C/mus301.*

4.5.6.3. *recQ5*

Drosophila has at least one other *recQ*-like gene that is most similar to human *RECQ5.* It appears to be produced in two isoforms. One set of mRNAs would generate a protein of about 54 kDa that consists of little more than the helicase core region. Another set of mRNAs encodes a protein more than double this size with an protracted C-terminus abundant in charged residues, as seen in many other *recQ* members *(153)* (Fig. 6). The human *RECQ5* transcript also exists in multiple forms capable of producing short or extended proteins *(153,157).* Drosophila RECQ5 is concentrated in the nucleus, compatible with a DNA helicase activity.

4.5.7. Drosophila Homologs of MRE11, RAD50, and NBS1

In *S. cerevisiae,* Mre11p, Rad50p, and Xrs2p proteins form a complex involved in the repair of DSBs. An analogous complex is formed in humans between hMRE11, hRAD50, and hNBS1 *(25)* (*see* Chapter 7). This complex localizes to foci at the site of DNA breaks where it may be involved in both enzymatic (end-processing) and damage-signaling functions *(104,121,170).* Null mutations of mouse *mMre11* and *mRad50* are lethal *(102,174).* Non-null mutations in human *nbs1* and *mre11* cause the severe chromosome-instability disorders Nijmegen breakage syndrome and Ataxia telangiectasia-like disorder, respectively *(25,106,162,171).*

Drosophila has *MRE11, RAD50,* and *NBS1* homologs, but no mutations in these genes have been reported. The sequences of Drosophila, human, and yeast MRE11 and RAD50 proteins are very similar. In contrast, the Drosophila NBS and hNBS1 proteins are rather diverged and they share very little resemblance to yeast Xrs2p. The similarity that they do share is limited to their N-termini in a region that constitutes a forked head-associated (FHA) domain (Fig. 7). The FHA domain is an amino acid sequence motif found in many proteins of diverse functions. A few FHA-containing proteins are involved in DNA damage-inducible cell-cycle checkpoints (e.g., Dun1p, Spk1p, Mek1p), but many have no apparent connection to DNA repair. The FHA domain is assumed to play a role in nuclear signaling *(71).* The Drosophila and human NBS proteins share an additional domain in common bordering the FHA domain. It includes a BRCA1 C-terminal (BRCT) domain, originally identified in the breast cancer-susceptibility gene BRCA1. Many DNA repair proteins and some cell-cycle checkpoint proteins contain BRCT domains (e.g., DNA ligases). This domain is thought to form an interface for protein-protein interactions *(12).* Human NBS1 is phosphorylated in response to ionizing radiation. This phosphorylation is ATM-dependant and is required

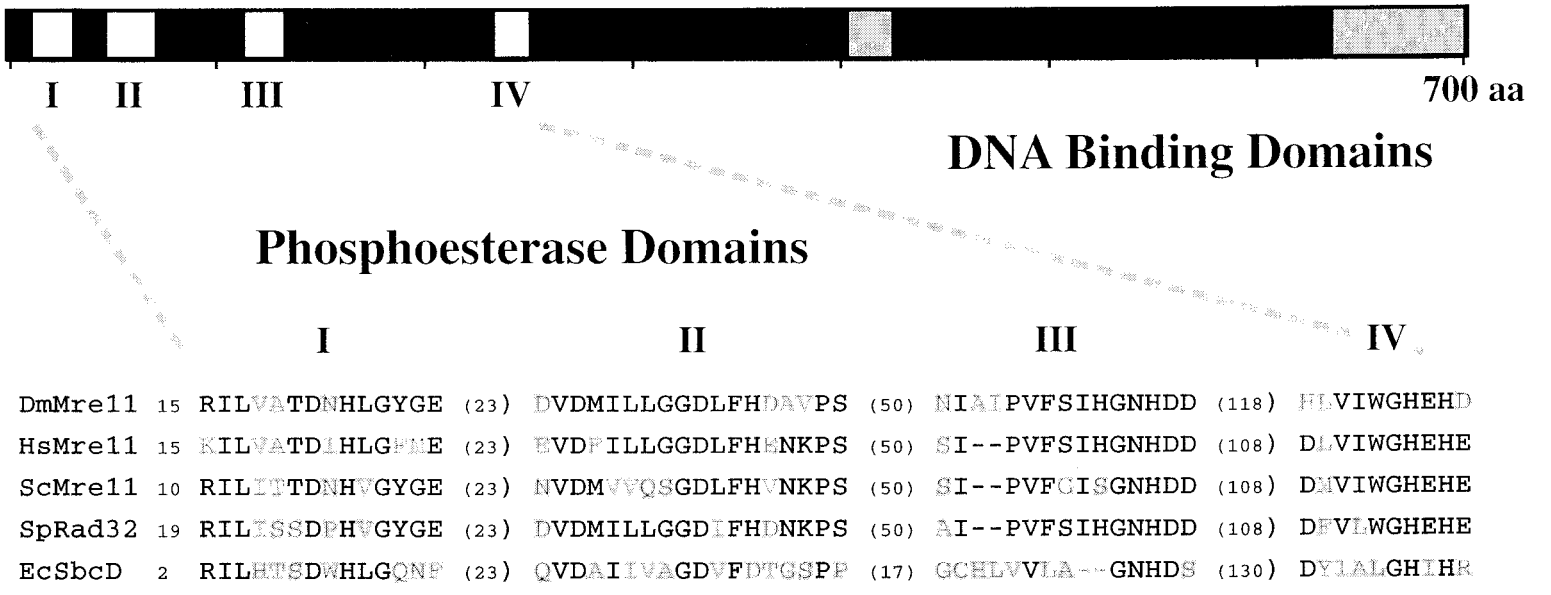


Fig. 7. Domain structure of MRE11 proteins. MRE11 proteins contain four conserved domains near the amino termini that are common to many proteins with phosphoesterase activity. Two apparent DNA binding domains are in the carboxy terminal halves. Also illustrated is an alignment of the amino acid sequence in the phosphoesterase domains from Drosophila, human, and yeast MRE11, as well as the more distantly related SbcD protein from *E. coli.*

for normal induction of an S-phase checkpoint *(98).* Drosophila *nbs* mRNA is found in both males and females and during most developmental stages, but it is most abundant in early embryos, pupae, and the adult head *(44).*

The Drosophila *mre11* gene was isolated using degenerate PCR. The MRE11 amino acid sequence is 36% and 29% identical to human and yeast homologs, respectively *(44).* MRE11 contains several distinct functional domains. Two domains involved in binding to DNA are highlighted in Fig. 8. Another domain contains phosphoesterase sequence motifs, which are required for MRE11 exonuclease activity and some but not all of the in vivo phenotypes of yeast and human *mre11* mutants.

The presumed sequence of Drosophila RAD50 is 29% identical to human RAD50 and 27% identical to yeast Rad50p. These proteins contain a large region of diverged sequence in their centers that may adopt a coiled coil structure, whereas the N- and C-termini are much more highly conserved. The N-terminal half of human RAD50 is able to bind to the BRCA1 protein *(176).* The structure of the Rad50 catalytic domain from a thermophilic bacterium suggests a mechanism that is driven by cycles of ATP-dependant dimerization and dissociation upon hydrolysis *(73).* Drosophila rad50 is expressed most highly in 0–8-hr embryos and moderately in late larval to adult stages *(44).*

4.5.8. p53 (Dmp53)

There have been rumors of a fly gene similar to human *p53* for several years *(39),* but it was only recently isolated *(20,75,130).* The fact that Drosophila possessed an intact signaling pathway capable of responding to human p53 was demonstrated earlier *(175).* Human *p53* expressed in Drosophila developing eye tissue was shown to cause severe apoptosis *(175).* The Drosophila homolog of p53 can bind to the same DNA targets as human p53 *(20,75,130).* Overexpression of *Dmp53* also induces apoptosis in flies *(130).* Dominant-negative mutant forms of DMP53 inhibit transactivation *(20,75)* and radiation-induced apoptosis *(20,130)* but do not appear to affect X-ray-induced cell-cycle arrest *(130).* Neither does overexpression of *Dmp53* seem to induce G1 arrest *(130).* Studies in the genetically tractable Drosophila model should provide new insight into the complexities of p53 function in cell-cycle control, DNA repair, and apoptosis. Some transcriptional targets of Drosophila p53 have been discovered *(20).*

4.5.9. Poly(ADP-Ribose)Polymerase

Poly(ADP-ribose)polymerase (PARP) plays an important regulatory role in DSBR in eukaryotes. Once a chromosome is broken, PARP quickly binds to the ends and begins to modify itself and other specific proteins in the vicinity by adding ADP-ribose residues until long, branched chains are attached. Many of the known PARP targets are DNA-binding proteins. Modification by PARP is believed to cause the dissociation of proteins from the DNA, clearing the region and thus facilitating DSBR. Activation of PARP causes rapid redistribution of many proteins within the nucleus. In Drosophila, PARP transcripts are highly abundant through the first half of embryogenesis and are distributed homogeneously except for the pole cells *(60).* The mRNA is also found at moderate levels in pupae and adults *(60).* Two classes of transcripts have been detected *(80,114).* Form I produces a full-length, active PARP enzyme, whereas form II lacks exon 5 encoding the auto-modification domain and appears to have no enzyme activity *(114).* The biological significance of form II is unknown though it can interfere with growth and development in cultured rat cells *(80).* Although no consensus cleavage site for a CED-3 like protease is found in fly PARP,

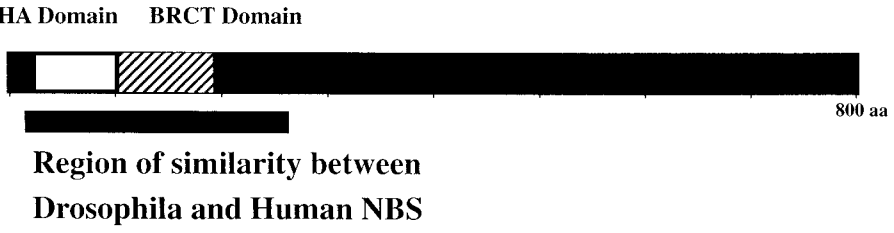


Fig. 8. Domain structure of NBS proteins. Only the first third of the human and Drosophila NBS proteins resemble each other. Within this region, both proteins share amino acid sequences matching the FHA domain consensus and the BRCT domain consensus.

it does appear to be processed during induced apoptosis *(136).* In Drosophila testes, PARP is activated in response to α-irradiation *(94).* PARP often forms foci or localizes to the nuclear rim in irradiated premeiotic and postmeiotic cells. In primary spermatocytes, PARP staining co-localizes with the discrete chromosomes at the periphery of the nuclei *(94).* Deletions of the *parp* gene lead to lethality late in embryogenesis *(114).* More subtle manipulations of PARP will be required to dissect its role in DSBR.

4.5.10. Other Repair Genes

Many studies have probed the function of Drosophila DSBR genes. A sample of these includes: the *mei-9* gene, a homolog of the yeast *RAD1* and human *XPF* genes *(154); mei-W68,* a homolog of the yeast meiotic endonuclease gene *SPO11 (111,112);* a *RAD51* homolog that has elevated expression in the ovaries *(2,110); Rrp1,* a gene encoding a combination AP endonuclease/3′ exonuclease similar to mammalian *APEX* genes *(147,148,168);* the *grapes* gene, which is homologous to human *CHK1 (46,159,164,165);* and *loki,* a gene encoding a serine/threonine kinase that is expressed in the ovary and early embryo and is similar to human *CHK2* and *S. cerevisiae DUN1 (128). mus304* confers mutagen sensitivity and is another checkpoint gene, but as yet is only known in flies *(21).* Dozens of other repair genes have been identified in flies but await experimental analysis. These include *FEN1, KU86, XRCC1, RAD21, RAD17, RAD1,* a *HUS1*-like gene, *RAD9,* DNA ligase I, DNA ligase III, DNA ligase IV, and genes with weaker matches to *XRCC2, RAD51, ATM, ATXR, FRAP,* and many others. Among DNA repair genes that are conspicuously absent from Drosophila homologs of *RAD52, RAD57,* and *RAD59,* and the MMR genes *MSH3, MSH4, MSH5, MLH2,* and *MLH3.* This explosion of enticing new leads to explore has ushered in a new era for research of DNA repair in Drosophila.

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9

Double-Strand Break Repair and Homologous Recombination in Mammalian Cells

Maria Jasin

1. INTRODUCTION

A paradigm shift has occurred over the last few years in the understanding of homologous recombination. It has long been known that DNA double-strand breaks (DSBs) in yeast are potent inducers of homologous recombination and that homologous recombination is the major pathway in yeast to repair DSBs (Chapter 16, Vol. 1). Compared with nonhomologous repair, homologous recombination has generally been considered to be inconsequential as a DSB repair pathway in mammalian cells. However, homologous repair can precisely restore the damaged DNA to its original sequence, suggesting that it should be a preferred pathway for repair, at least under some circumstances. Recently, direct examination of repair products in mammalian cells has demonstrated the importance of homologous recombination during the repair of DSBs. Supporting this conclusion has been the identification of DNA repair defects in mutant cell lines and the construction of mouse knockouts of genes implicated in homologous recombination. This chapter discusses basic parameters of DSB repair by homologous recombination in mammalian cells and emerging evidence for the involvement of various proteins in the repair process.

2. TOOLS TO STUDY CHROMOSOMAL DSB REPAIR

Experiments that address the mechanism of DSB repair generally begin with the introduction of one or more DSB(s) into a target molecule. DSBs can be introduced into either plasmids or chromosomal DNA by a number of techniques. Although the focus of this chapter is on the introduction of a DSB at a defined site in mammalian chromosomes, it is important at the outset to contrast this approach with other experimental approaches.

2.1. Chromosomal vs Plasmid DSB Repair

There is an extensive literature on the analysis of DSB repair in mammalian cells using plasmid substrates *(134).* These substrates are cleaved in vitro by restriction enzymes, introduced into cells, and then recovered either as plasmids or integrated into genomic DNA to determine the mode of repair. However, the relevance of plasmid DSB repair to

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chromosomal DSB repair is uncertain because plasmid substrates are introduced without chromatin proteins and are rapidly degraded after transfection *(80).* Not surprisingly, substantial differences between plasmid and chromosomal homologous recombination products have been reported *(150).* This contrasts with studies of V(D)J recombination in which plasmid substrates appear to faithfully recapitulate aspects of DSB repair during endogenous antigen-receptor gene rearrangement *(77).* The plasmid substrates in this case replicate and become chromatinized before DSBs are introduced into them by the recombinase proteins. For most other studies, especially for homologous recombination, direct examination of chromosome DSB repair is more biologically relevant.

2.2. Nonspecific Agents for Introducing DSBs into Genomic DNA

Various nonspecific agents have been used to introduce DSBs into mammalian genomes, including ionizing radiation (IR) and radiomimetic drugs. Although IR causes a variety of lesions, e.g., single-strand breaks and base damage, DSBs have been deduced to be the toxic lesions (see Chapter 25, Vol. 2). IR and radiomimetic drugs are useful for understanding the global response of a cell to DNA damage and for the identification of repair mutants. However, the molecular analysis of the repair of such breaks is difficult, because the position of the break site is unknown and multiple lesions are introduced. Alternatively, restriction enzymes provide sequence specificity, as well as a defined type of DSB, i.e., a 5′ or 3′ overhang or blunt end. Restriction enzymes are introduced by electroporation or streptolysin O poration of cells *(14).* Although the potential number of cleavage sites for restriction enzymes is numerous (e.g., 1.5×10^6 sites per genome for a 6 bp recognition site), the enzymes can be titrated to introduce a limited number of breaks to allow cell survival. As a result of the cleavage specificity, it is possible in some cases to infer the chromosomal location of the DSB and to determine the molecular mechanism of repair. For example, mutations in the *aprt* gene have been selected after restriction-enzyme electroporation and then mapped to determine if their location corresponds to the location of a cleavage site for the introduced restriction enzyme *(108).* This approach has been useful in studies of mutagenic nonhomologous repair because loss of function mutations can be selected at a gene such as *aprt.* However, the utility of this approach is limited to a few such selectable markers and the sites of cleavage are difficult to predict. As with IR and radiomimetic drugs, multiple DSBs are introduced per cell, making unclear what effect the introduction of global damage has on the repair of one particular DSB. A large number of DSBs may induce a DNA damage response that would not normally be found in cells with one or a few breaks in the genome.

2.3. The Rare-Cutting I-SceI Endonuclease

To overcome the limitations of more general DNA damaging agents, a broadly useful system to study DSB repair has been developed that relies on the expression of an endonuclease with few (or no) endogenous sites in mammalian genomes *(63).* The endonuclease, I-*Sce*I, is derived from *Saccharomyces cerevisiae* and studies with it parallel those in yeast that use the HO endonuclease (Vol. 1, Chapter 16). I-*Sce*I is from a class of endonucleases involved in intron homing *(6),* its normal role being to initiate a gene conversion event by the introduction of a DSB into the mitochondrial rDNA locus *(34).* I-*Sce*I has been used in mammalian cells to introduce DSBs into genomic DNA,

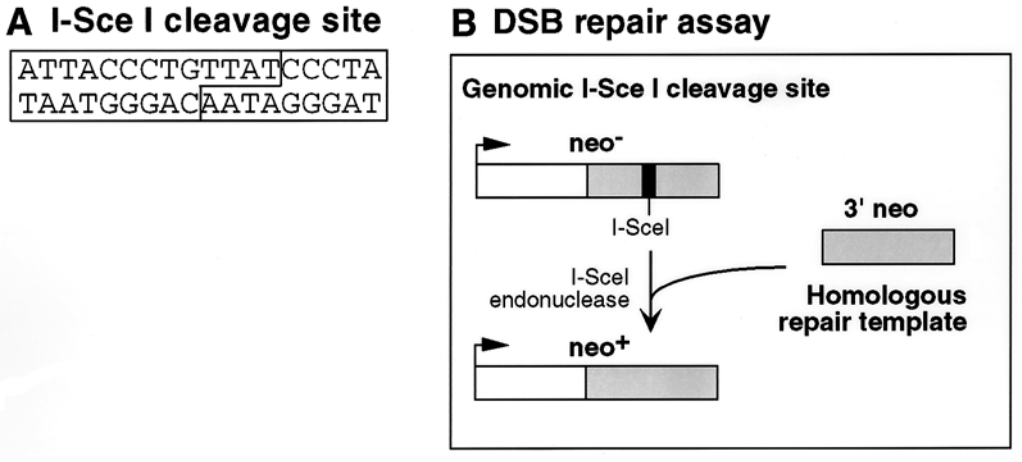


Fig. 1. DSB repair assay system for mammalian cells. **(A)** The 18 bp I-*Sce*I cleavage site *(25).* I-*Sce*I endonuclease cleavage produces a 4 base 3′ overhang. **(B)** The I-*Sce*I DSB repair-assay system. This system can be used to assay homologous or nonhomologous repair *(117).* The I-*Sce*I site is cloned into a selectable marker gene, in this case the *neo* gene, disrupting its function. The *neo*⁻ gene is integrated into the genome of mammalian cells. I-*Sce*I endonuclease is expressed in vivo from a transfected I-*Sce*I expression vector and cells that contain a *neo*⁺ gene can be selected after DSB repair. In this case, DSB repair occurs from a homologous *neo* fragment that contains the 3′ end of the gene (3′ *neo*) and can correct the I-*Sce*I site mutation. To analyze different types of recombination, the homologous fragment can be transfected (gene targeting), linked to the *neo*⁻ gene (intrachromosomal recombination), or unlinked on another chromosome (interchromosomal recombination). NHEJ can be assayed in nonselected cells as those that have lost an intact I-*Sce*I site.

and, like its natural role in the mitochondrial gene conversion event, it is not believed to play any role in the subsequent repair process after the initial introduction of the DSB.

The scarcity I-*Sce*I cleavage sites in complex genomes is owing to the length of its recognition site. The site is 18 bp and is nonpalindromic, having a 4 base 3′ overhang following cleavage (Fig. 1A). Mutagenesis studies of the recognition site have shown that the identities of most nucleotides within the 18 bp are essential for efficient cleavage activity *(25).* Not surprisingly then, the yeast nuclear genome contains no I-*Sce*I cleavage sites *(143).* For mammalian cells, an 18 bp site would statistically be expected to occur once every 20 mammalian genomes, although the small amount of degeneracy in the site makes conclusive determination of the number of sites difficult. The coding region for I-*Sce*I has been modified to a universal code in which mitochondria-specific codons are replaced with more standard codons, allowing the endonuclease to be expressed in *E. coli* and other cell types *(24).* The enzyme has been purified *(95)* and biochemically charactacterized *(105),* and it is sold commercially (Boehringer Mannheim). When I-*Sce*I has been used in yeast, similar kinetics and products of repair have been obtained as when DSBs are introduced by HO endonuclease *(38,112).*

2.4. I-SceI Expression in Mammalian Cells: In Vivo DSBs

A number of I-*Sce*I expression vectors have been constructed for use in mammalian cells. In some vectors, the I-*Sce*I coding region (universal code version) has been fused

at the N-terminus to three copies of a nuclear localization signal (nls) and an HA epitope tag *(see 32).* The nls may assist in translocation of the endonuclease to the nucleus although it is clearly not essential *(22).* Without an nls, the 235 amino acid protein is presumably small enough to diffuse passively through nuclear pores. Several promoters have been used to express I-*Sce*I, including human cytomegalovirus *(22,122),* mouse phosphoglycerate kinase *(32,131),* and chicken β-actin *(118),* as well as meiosis specific promoters (P.J. Romanienko and M. Jasin, unpublished results). Consistent with a lack of I-*Sce*I sites in the mouse genome (or at least a small enough number of sites that I-*Sce*I-generated DSBs are efficiently repaired), constitutive expression of I-*Sce*I is not toxic to mouse cells *(121)* and mice expressing I-*Sce*I during meiosis are fertile (P.J. Romanienko and M. Jasin, unpublished results).

DSBs were first detected in vivo using plasmid substrates, where it was found that I-*Sce*I cleavage would stimulate recombination between adjacent repeats, similar to when plasmids were pre-cleaved in vitro by restriction enzymes *(121).* Recombination between plasmid repeats is believed to occur by the single-strand annealing pathway *(83).* In this pathway, the DSB at opposite ends of each repeat provides an entry site for an exonuclease, so that resultant single-strands are able to anneal at exposed regions of homology. This is considered a nonconservative pathway because sequence information between the annealed homology regions is lost. Although this pathway is relevant to some forms of chromosomal recombination (*see* Subheading 4.), it probably predominates in plasmid recombination because plasmid DNA is readily degraded by cellular nucleases upon transfection.

3. CHROMOSOMAL DSBs AND RECOMBINATIONAL REPAIR DURING GENE TARGETING

3.1. Chromosomal DSBs Generated by I-SceI

Plasmid-recombination experiments demonstrated that I-*Sce*I was functional within mammalian cells, anticipating the advance in the field that came when I-*Sce*I was applied to chromosomal DSB repair studies *(22,122).* One approach for studying chromosomal DSB repair is illustrated in Fig. 1B. The I-*Sce*I cleavage site is integrated into the genome by standard transfection protocols, typically in the context of a defective selectable marker such as the neomycin phosphotransferase *(neo)* gene. An endonuclease expression vector is subsequently transfected into the cells to transiently express I-*Sce*I and a DSB is introduced into the chromosomal site within the first 24 h or so after transfection. Repair presumably occurs rapidly thereafter. Thus far, synchronous cleavage in 100% of the cells has not been achieved, limiting direct analysis of processing of the broken chromosome ends. However, DSB repair products have been readily recovered by selecting for restoration of the previously defective selectable marker after a particular type of repair *(22,32,81,99,118,122,123,131,140).* Individual repair events have also been examined after random cloning after I-*Sce*I cleavage to study repair without bias to the particular pathway that is used *(79).* Alternatively, analysis of unselected repair products has been performed on populations of cells using PCR *(79).*

3.2. DSBs Induce Recombination 100- to 1000-Fold

If homologous recombination mechanisms in mammalian cells are similar to those in *S. cerevisiae,* a single DSB in genomic DNA would be expected to induce recombination.

This was first tested in gene-targeting experiments, which allow the introduction of mutations into genomes *(15).* Gene targeting in mammalian cells is inefficient both in absolute frequency and in comparison to random integration of DNA. Early studies demonstrated that a DSB in a transfected plasmid would significantly increase homologous recombination with the chromosome *(57,65,66).* However, in recombination models, the recombining partner that contains the DSB is normally the recipient of genetic information (e.g., *49,137*), suggesting that a more substantial induction of gene targeting would occur if the DSB were in the chromosomal target rather than the transfected plasmid.

This was tested by introducing a DSB into the genome with I-*Sce*I during the transfection of a homologous repair template (Fig. 1B). The frequency of gene targeting was at least two to three orders of magnitude higher with a DSB at the target locus than without *(22,122),* conclusively demonstrating that a DSB in a mammalian genome is highly recombinogenic. Because the chromosome was converted at the DSB site to the sequence of the incoming plasmid DNA, the mechanism of recombination appeared to be different from the nonconservative single-strand annealing mechanism discussed for plasmid recombination (*see* Subheading 4).

The length of homology shared by the plasmid and chromsome in these experiments was relatively short. In one case, it was only 700 bp, short enough that spontaneous recombinants were extremely rare *(122).* More recent experiments have demonstrated that DSB-induced recombinants can be detected with as little as 69 bp of homology, despite the fact that the 18 bp I-*Sce*I site interrupts this very short length of homology (C. Richardson, J. Winderbaum, and M. Jasin, unpublished results). The I-*Sce*I cleavage site is cleanly removed from the chromosome during recombination and converted to the sequence of the incoming DNA. By modifying the targeting fragment, it has been possible to introduce single bp changes into the chromosome at frequencies approaching 0.1% of transfected cells *(35,122).* Expression cassettes for foreign genes have also been introduced into the genome in this manner *(22).* Some of these experiments were performed in embryonic stem (ES) cell lines *(35,131),* which are used for creating mutant mice *(15).* Targeting has been performed with linear or circular DNA. With linear fragments, targeting events have been recovered in which homologous recombination occurred at both ends of the fragment whereas other events had a homologous event at one end and a nonhomologous rejoining event the other end *(122).* The proportion of these two types of events suggested that homologous recombination was nearly as frequent as nonhomologous repair of DSBs (*see* Subheading 5). Interestingly, the proportion of targeting events with coupled nonhomologous events was much lower in ES cells *(131).*

3.3. DSB-Induced Gene Targeting at Endogenous Loci

The recombinogenicity of DSBs in ES cells suggests that DSB-promoted gene targeting may be useful for efficiently altering the mouse genome. One round of gene targeting by conventional methods is necessary to introduce the I-*Sce*I site at the locus to be modified. If this first round of targeting brings in a marker that can be selected against, subsequent targeting events should be readily selectable and occur at high frequency upon break induction *(63).* The utility of this approach requires that the recombinogenicity of DSBs is not limited to randomly integrated I-*Sce*I sites, but that it is also found at I-*Sce*I sites targeted to specific chromosomal loci. This has indeed been

found at the *pim*-1 locus on chromosome 17 *(118)* and the *hprt* locus on the X chromosome *(32)*. At the *hprt* locus, gene targeting frequencies of 1% have been achieved using efficient calcium phosphate transfection protocols. In this case, the targeting fragment was 5.5 kb and included small palindromic insertions *(32)*. One case of a "gene exchange" has been performed at an endogenous locus in ES cells in which an ezrin gene mutant was integrated at the villin locus at high efficiency using DSB-promoted gene targeting *(23)*. This type of approach may provide a controlled means of expression of introduced genes, as described for the lox/Cre recombination system *(43)*.

4. RECOMBINATIONAL REPAIR: INTRACHROMOSOMAL RECOMBINATION

The ability of a chromosomal DSB to stimulate gene targeting suggested that a DSB would also induce recombination between two homologous chromosomal sequences. This was first addressed by examining recombination between closely linked homologous sequences in the mammalian genome, i.e., intrachromosomal recombination. Substrates to measure intrachromosomal recombination typically have two homologous sequences oriented as direct repeats (Fig. 2). The direct repeats, consisting of differentially mutated selectable marker genes and located within a few kb of each other, can recombine either by intrachromatid or sister chromatid recombination. Spontaneous recombination between such repeats occurs at a low but detectable frequency, generally 10^{-4} to 10^{-6} *(84,132)*. Although described as "spontaneous," it is likely that a lesion in the chromosome triggers these recombination events. DSBs introduced by I-*Sce*I increased recombination between the direct repeats *(32,79,81,123,140)*. As with gene targeting, the induction of recombination was substantial, usually two to three orders of magnitude, resulting in a recombination frequency of $\geq 10^{-2}$ to 10^{-3}.

Some direct-repeat recombination systems can differentiate between two different products, a deletion or a simple gene conversion. In one system, a deletion is the only repair product that can be selected owing to the marker-gene configuration *(32)*. In this case the I-*Sce*I cleavage site is between the 5′ and 3′ portions of the *hprt* gene and a deletion at the repeats is required to produce *hprt*$^+$ colonies. In three other systems, either recombination product can be selected *(79,123,140),* as shown in Fig. 2. The relative proportion of the two products differs in the three systems. In Taghian and Nickoloff *(140),* simple gene conversions predominate (97%), whereas in Liang et al. *(79),* deletion products predominate, with gene conversions only 25–30% of total recombinants. Both of these systems are composed of *neo* gene repeats separated by a spacer and the experiments were performed in hamster cells. The flip-flop in the relative recovery of the two recombination products may reflect differences in the lengths of the repeats or in the composition or lengths of the spacer sequences. The third system gives results intermediate between these two *(79,123,140)*.

The relative frequency of the deletion and gene-conversion products is important both mechanistically as well as for the consequence to the cell. Conversion events without crossovers are less mutagenic because deletions, by definition, result in loss of sequence information. In considering mechanisms for these events, single-strand annealing, as described earlier for plasmid recombination, would give rise to deletion events. Alternatively, DSB repair by a classical conservative recombination model proposed for yeast predicts an equal frequency of crossover (deletion) and noncrossover

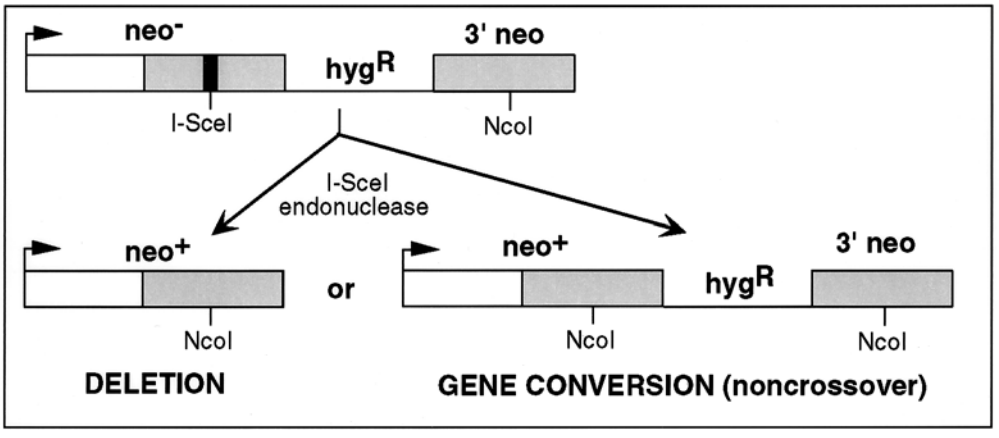


Fig. 2. Direct repeat recombination substrate for DSB repair. Two defective *neo* genes are in direct orientation. DSB-induced recombination results in two different types of products, a deletion or a noncrossover gene conversion. In both cases, the DSB site is converted to an *Nco*I restriction site. The deletion product can occur by single-strand annealing or by a gene conversion with an associated crossover (*see* text).

events *(137).* Another conservative recombination model has also been proposed in yeast and other organisms in which DSB-promoted recombination is tightly coupled to replication, resulting in a predominance of noncrossover gene conversion events (*see* Subheading 8.4. and *59*). This latter model, at least in broad outline, would be consistent with the results of Taghian and Nickoloff *(140),* whereas the results of Liang et al. *(79)* are best explained by the use of two different recombination pathways. Single-strand annealing could give rise to the predominance of deletion products and noncrossover conservative recombination could give rise to simple gene-conversion events.

Most DSB-promoted recombination experiments have been performed in rodent cells. In particular, hamster CHO-K1 cells *(79,81,123,140)* and mouse 3T3 *(122),* EC *(22),* and ES cells *(32,35,99,118,131)* have all been utilized with broadly similar results. Direct repeat recombination has also been examined in human cells, where it has been found to be stimulated by a DSB (A.J. Pierce and M. Jasin, unpublished results; *13*). An alternative to I-*Sce*I, PI-*Sce*I endonuclease (PI for protein intron; *see 48*), was used to introduce DSBs in human cells by Brenneman et al. *(13).* In this case, the endonuclease was directly electroporated into cells rather than expressed from an expression vector. The induction of recombination was only 10-fold, possibly owing to inefficiencies of enzyme electroporation. Because PI-*Sce*I endonuclease cleaves inefficiently and also binds avidly to one end after introducing a DSB in vitro, it is unlikely that this endonuclease will have general utility.

5. RELATIVE CONTRIBUTION OF HOMOLOGOUS RECOMBINATION AND NONHOMOLOGOUS END-JOINING IN MAMMALIAN DSB REPAIR

Three lines of evidence suggested that nonhomologous repair is the dominant DSB repair pathway in mammalian cells (Vol. 2, Chapter 16). When comparing homologous targeting and random integration of transfected DNA, random integrations were found

to dominate, in some cases by several orders of magnitude *(15).* Secondly, linearized plasmids transfected into cells were found to be readily rejoined by nonhomologous end-joining (NHEJ) *(120).* One study that directly compared NHEJ and homologous recombination of plasmid substrates nevertheless found that both types of repair occurred at a similar frequency *(67).* However, because plasmid recombination may not directly reflect chromosomal recombination, these studies were not considered to be conclusive. A third line of evidence came from cell-mutant studies. The first IR-sensitive cell mutants that were examined were found to be defective in NHEJ as demonstrated by their deficiency in V(D)J recombination *(139).* (For more information on repair proteins in V(D)J recombination, *see* Chapter 11.) Because these cells were found to be proficient at homologous recombination between plasmids *(81),* a role for homologous recombination in DSB repair was not obvious.

To determine if homologous recombination is a major pathway for repairing chromosomal DSBs in mammalian cells, DSBs were introduced by the I-*Sce*I endonuclease in a direct repeat recombination substrate (Fig. 2). Individual products of repair were analyzed in clones following nonselective growth and populations of repair products were analyzed by using polymerase chain reaction (PCR) *(79; see also 68a).* Both approaches indicated that 30–50% of DSB repair occurred by homologous recombination, demonstrating that homologous recombination is a major DSB repair pathway in mammalian cells. A portion of nonselected cell clones and the PCR products maintained the I-*Sce*I site in these experiments. This could indicate incomplete cleavage in the transfected cell population or retention of the I-*Sce*I site after repair. Restoration of an intact I-*Sce*I site by DSB repair could occur by either precise ligation of the I-*Sce*I overhangs *(83a)* or by equal sister chromatid recombination, as long as the I-*Sce*I site on the sister chromatid is not also cleaved. As yet, these possibilities cannot be distinguished. Nevertheless, they should be considered when evaluating the contributions of various pathways to DSB repair. In yeast, recent indirect evidence suggests that precise rejoining of HO endonuclease-induced DSBs may comprise as much as 50% of chromosomal DSB repair *(22a).*

In several studies using the I-*Sce*I system, NHEJ products have been sequenced *(79,83a,88,122,123).* Many of the products showed rejoining of the ends with loss of 1 bp or a few bp from the I-*Sce*I overhang and adjacent sequences. The deletions were often at short-sequence overlaps, as if the broken ends aligned prior to repair. Insertions of 1 bp were also recovered *(122).* Larger deletions and insertions were obtained less frequently. Larger deletions were found to have similar types of breakpoint junctions as the smaller deletions *(123).* Insertions came from a variety of sources, including single-copy DNA, repetitive elements in the genome, and the transfected I-*Sce*I expression vector *(79,108,109,123).* Microsatellite $(GT)_n$ repeats in a few instances have been found directly inserted at the chromosome break site *(79,108),* raising the possibility that DSB repair mechanisms contribute to their spread in the genome *(79).* As with the deletions, some insertions occurred at short-sequence overlaps, indicating pairing of a few nucleotides between the broken end and the sequence to be inserted.

6. REPAIR OF DSBs AND SISTER CHROMATID RECOMBINATION

The robust stimulation of homologous recombination between two chromosomal sequences suggests a physiological role for recombination in DSB repair. Recombination

between closely linked direct repeats in model systems can occur between two repeats on the same chromatid or, after DNA replication, between two repeats on sister chromatids. This suggests that sister chromatids and/or natural sequence repeats could be used as repair templates. The large number of repetitive elements in mammalian genomes suggests a role for sequence repeats, although repetitive elements can often be quite divergent. (The effect of sequence divergence on DSB repair is discussed in Subheading 9.) Alternatively, sister chromatids are ideal homologous repair templates because they are identical to each other and they are held in close proximity until anaphase. Sister chromatids have been found to be preferred recombination partners for spontaneous recombination in yeast *(70)* and in mammalian cells *(11).* This section discusses evidence for sister chromatid recombination during DSB repair in mammalian cells.

6.1. S-Phase Radiation Resistance

Classical radiation studies have suggested a role for sister chromatids in DSB repair because cells irradiated at different points in the cell cycle show different sensitivities to IR *(142).* Using drug synchronization or elutriation, it has been found that cells in S phase can be fivefold more resistant to killing than cells in G1, depending on dose. The S-phase radiation resistance suggests that the presence of sister chromatids protects cells from killing by acting as a homologous repair template for DSB repair. However, it is also possible that expression of repair proteins is induced during DNA replication. Cells in S phase then would more efficiently repair lesions in DNA but by the same nonhomologous mechanisms that are used at other times in the cell cycle.

6.2. Direct Repeat Recombination as a Model for Sister Chromatid Recombination

Sister chromatid recombination can restore a damaged chromatid to its original sequence. This type of event is considered to be "equal" because it occurs between equivalently positioned sequences on sister chromatids. As a result of the precise nature of the repair, such events go undetected. However, unequal sister chromatid recombination can be detected by arranging repeats opposite to that shown in Fig. 2, so that 3′ *neo* is upstream of the *neo* gene. In this case, unequal sister chromatid recombination can create a *neo*$^+$ product that is part of a *neo* gene triplication. DSB-promoted recombination events leading to triplications have been readily observed with this substrate *(68a,68b),* conclusively demonstrating a role for sister chromatids in DSB repair in mammalian cells, and consistent with a role for sister chromatid recombinational repair in S-phase radiation resistance. Although it is still possible that increased expression of proteins involved NHEJ contributes to S-phase radiation resistance, NHEJ mutants show normal levels of survival in S phase *(47),* arguing against a strong contribution of NHEJ repair to S-phase radiation resistance.

7. RECOMBINATION BETWEEN HOMOLOGOUS CHROMOSOMES

7.1. Homologs as Potential Templates for Recombinational Repair

Homologs, like sister chromatids, are potential homologous repair templates for chromosomal sequences with the exception of non-pseudoautosomal regions of the X-Y pair. Although homologs may be somewhat diverged, unlike sister chromatids, they are present throughout the cell cycle and, therefore, provide homology in mitotic cells

at any stage of the cell cycle as well as in nondividing cells. One key difference between homologs and sister chromatids is proximity. Sister chromatids are attached to each other by cohesion proteins until mitosis *(93).* It is believed that cohesion proteins are assembled during DNA replication and disassemble at the metaphase/anaphase transition. With the exception of the specialized homolog pairing that occurs during meiosis *(106),* homologs, despite their sequence homology, are generally not any closer to each other than to heterologs *(40).* There may be exceptions to this, for example, the region of chromosome 15 in humans which is subject to parental imprinting *(74).* Therefore, chromosome organization in the nucleus does not appear to be random, and during the G1 to G2 transition there is extensive chromosome movement *(40).* In addition, homologous recombination enzymes may promote a genome-wide homology search, as occurs during yeast meiosis, bringing homologs into close proximity to allow the repair of damaged DNA.

7.2. Evidence for Recombination Between Homologs

Evidence for mitotic recombination between homologs has come from analysis of loss of heterozygosity (LOH) in tumor cells and in model systems *(64).* LOH results in the genetic information of a particular locus or chromosomal region being derived from only one parent. This can unmask deleterious mutations, as seen with tumor-suppressor genes. LOH can occur by many mechanisms but somatic recombination of homologs has a particularly important contribution *(19).* LOH arising from recombination between homologs has also been observed in normal tissues where it can encompass large portions of chromosome arms *(64).* It is unknown whether the frequency of LOH is elevated in tumor tissues, or whether normal cells that undergo LOH of tumor-suppressor genes gain a selective advantage.

LOH can be detected by loss of function of a marker that starts heterozygous but becomes homozygous. Recombination can lead to LOH if a crossover occurs between the centromere and the marker locus at G1 and the two mutant loci cosegregate in the next mitosis. The frequency of these events in normal tissues was found to be as high as 10^{-4} to 10^{-5} *(64).* Recombination between homologs was also detected in a cell line that is a compound heterozygote for mutations at the *TK* locus. Restoration of a TK^+ gene by recombination in this case required that recombination occurred between the two closeby *TK* mutations. TK^+ revertants were selected and those arising from homolog recombination were identified by LOH of linked markers at a frequency of approx 10^{-9} *(9).* In a follow-up study, it was demonstrated that IR can induce recombination between the *TK* genes in a dose-dependent manner *(8).*

The induction of homolog recombination by IR suggests that a DSB may be the lesion that initiates recombination. Because IR causes multiple types of DNA damage, it is also possible that IR activates the recombination machinery. To determine directly if a DSB induces recombination between homologs, the I-*Sce*I system has been utilized in mouse ES cells *(99).* An I-*Sce*I site was targeted to one allele of chromosome 14, and a DSB was introduced by transient expression of the I-*Sce*I endonuclease. In this system, recombination between homologs at the locus on chromosome 14 was increased two to three orders of magnitude by a DSB, to approx 10^{-5} to 10^{-6}. Homolog recombination in these experiments was verified by LOH of a marker a few kb downstream of the DSB. Polymorphisms were not available further downstream to determine a further

extent of gene conversion. No evidence was obtained for crossing-over between homologs in these experiments, although the lack of polymorphisms does not allow an unequivocal assignment of crossovers and noncrossovers for all recombinants. Interestingly, although the fold-increase is similar to that found with direct repeats, the absolute frequency of homolog recombination was approx three orders of magnitude lower *(99)*. Thus, homologs do not appear to be preferred or frequent repair templates.

8. RECOMBINATION BETWEEN HETEROLOGOUS CHROMOSOMES

8.1. Heterolog Recombination and the Risk of Translocations and Other Chromosomal Abnormalities

Mammalian cells have large numbers of sequence repeats dispersed throughout their genome. Recombination events between repeats on the same chromosome would lead to genome scrambling, including deletions and inversions, if they were resolved as crossovers. If crossovers involved repeats on two different chromosomes (i.e., heterologs), chromosomal translocations or acentric/dicentric chromosome pairs would result. Translocations would occur if the two repeats were oriented in the same direction relative to their respective centromeres. Acentric/dicentric chromosome pairs would result if the repeats were oriented in opposite orientations relative to their respective centromeres, leading to loss of the acentric chromosome and breakage/fusion/bridge cycles involving the dicentric chromosome in dividing cells, unless one of the centromeres becomes inactivated.

8.2. DSB-Induced Heterolog Recombination

To determine if sequence repeats on heterologs can be used as repair templates, two differentially mutated *neo* genes were targeted to chromosomes 17 and 14 in mouse ES cells, with the *neo* gene on chromosome 17 containing an I-*Sce*I site *(118)*. Spontaneous recombination between the *neo* loci on the two different chromosomes was extremely low ($<10^{-9}$) but DSB-induced recombination was readily detectable, occurring at least three orders of magnitude more frequently (10^{-6}). Interestingly, the frequency of DSB-induced recombination between heterologs was only slightly below that for homologs *(118)*. This is consistent with the observation that homologs are not generally any nearer to each other than heterologs in the mammalian nucleus *(40)*.

8.3. Translocation Suppression During DSB-Induced Recombination

The repair products of DSB-induced heterolog recombination were examined to determine if crossovers occurred. The 200 recombinants that were analyzed arose exclusively from gene-conversion events, with no evidence of translocations or other chromosomal abnormalities *(118)*. The majority of events (98%) were simple gene-conversion events confined to the repeated region, with no overall change to the architecture of the locus. In these events, a small amount of sequence information was transferred from the unbroken chromosome to the broken chromosome. The remaining events appeared to have transferred a larger amount of sequence information, extending downstream of the homology. In these events, NHEJ was predicted to complete the recombination event.

The crossover suppression that is seen in heterolog recombination events contrasts with the observation of translocations by nonhomologous mechanisms in other systems *(108,151)*. For example, reciprocal translocations with junctions reflecting NHEJ have

been induced at the *aprt* locus in growth-arrested hamster cells by treatment with bleomycin *(151).* Presumably, two contemporaneous chromosome breaks were improperly rejoined to give rise to the reciprocal recombination products *(151),* contrasting with the single DSB induced by I-*Sec*I in the heterolog recombination experiments. It is possible that translocations are more likely when a cell confronts multiple lesions. Alternatively, homologous repair may in general be less mutagenic than NHEJ and play a greater role in protecting the cell from genome rearrangements. Evidence to support both of these hypotheses has recently been obtained, in which repair of two chromosomal DSBs has been found to lead to frequent translocations using NHEJ and SSA repair mechanisms *(117a).*

8.4. Replication-Based Recombination Mechanisms

The lack of translocations thus far observed in DSB-induced heterolog recombination is not consistent with a recombination model in which both crossover and noncrossover events are equally probable *(137).* Instead the results support a model in which noncrossover gene conversions are the predominant outcome of recombination (*see 49* and refs. therein). In this model, recombination is coupled to repair DNA synthesis. In one version (Fig. 3), the 3′ end from one side of the DSB invades the homologous sequence on the other chromosome and primes DNA synthesis, using the homologous sequence as a template. This leads to a restoration of sequence information originally present at the DSB site. The extended strand can dissociate and then reanneal to the homologous sequences on the broken chromosome. If the synthesis extends past the homology, NHEJ may complete the repair event. Recent evidence lends strong support for this model *(117b).* Although crossover events can be accommodated in this model (e.g., *39*), the majority of events are expected to be noncrossover gene-conversion events. The template remains unchanged during this type of repair.

Similar models have been proposed for yeast *(59), Drosophila (49),* and *Ustilago (39)* recombination, and in bacteriophage T4 replication *(41).* Other evidence for this type of mechanism in mammalian cells comes from experiments in which a gene-targeting vector has been demonstrated to prime synthesis from the target locus, leading to a correction of the mutation in the targeting vector *(124)* and from gap repair experiments of LINE-1 elements *(7).* The predominance of noncrossover gene conversion events in somatic cells contrasts with recombination in meiosis. Crossing-over during meiosis is essential for proper chromosome segregation at the reductional division. However, no such requirement holds in mitotic cells, which only undergo equational divisions. Importantly, noncrossover recombination mechanisms in mitotic cells safeguard the cell against genome scrambling. Other controls may exist in meiosis to prevent deleterious genome rearrangements.

8.5. Relative Contributions of Sister Chromatid, Homolog, and Heterolog Interactions During DSB-Induced Recombination

DSBs induce sister chromatid, homolog, and heterolog recombination each by approx two to three orders of magnitude. Clearly different, however, is the absolute level of recombination of these three substrates, with sister chromatid recombination occurring two to three orders of magnitude more frequently than either homolog or heterolog recombination *(68a).* Thus, in mammalian cells as in yeast, sister chromatids are

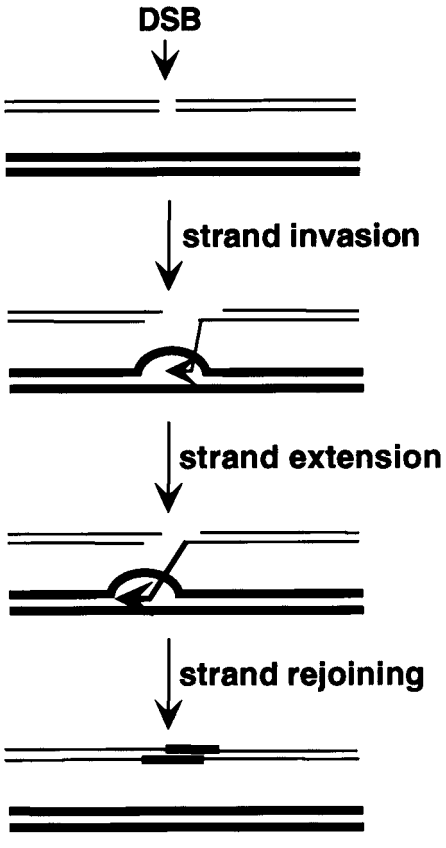


Fig. 3. Replication-based recombination. After a DSB occurs in one duplex, one of the 3′ ends that is produced invades a region of homology and primes DNA synthesis. A "D-loop" is formed by the strand invasion. The invaded homology region serves as a template for repair DNA synthesis, resulting in migration of the D-loop. The extended strand then reanneals to the other broken strand and the duplexes are sealed by ligation. This is the simplest version of several models that have been proposed. *See* ref. *(118)* and refs. therein.

preferred substrates for recombinational repair of DSBs, probably owing to their close proximity.

Interestingly, sister chromatid recombination induced by a DSB also has a predominately noncrossover outcome, like interchromosomal recombination *(68a,118).* These results parallel those obtained in spontaneous recombination experiments. Spontaneous recombination was measured at an immunoglobulin locus in a mouse hybridoma cell line. Recombination between direct repeats occurred at a rate of 10^{-4}/cell generation *(4),* somewhat higher than that found in other systems. However, recombination between homologs occurred orders of magnitude less frequently, about 10^{-8}/cell generation *(130)* and recombination between unlinked sequences likely to be on different chromosomes was also significantly less frequent, about 10^{-7}/cell generation *(3).* Thus, although spontaneous recombination is much lower than DSB-promoted recombination, the relative contribution of sequences located in different chromosomal positions to recombination rates is broadly similar.

9. SEQUENCE DIVERGENCE AND DSB REPAIR

9.1. Repetitive Elements in Mammalian Genomes

The large component of repetitive elements in mammalian genomes together with the observation of recombination between direct repeats raise the possibility that the endogenous sequence repeats are used for homologous repair of chromosome DSBs. In humans repetitive elements constitute fully one-third of the genome, with *Alu* elements, the most abundant class of dispersed repeat sequences, estimated at 10^6 copies *(125).* Complicating an analysis of recombination between repetitive elements is that elements within each class display heterogeneity in terms of length and degree of identity. *Alu* elements, for example, have 70–98% sequence identity with the consensus *Alu* sequence. A few examples of *Alu-Alu* recombination have been reported that are associated with the etiology of various diseases *(28).* Although these recombination events clearly involve sequence homology, it is not clear if they are *bona fide* homologous recombination events involving Rad51 and other recombination proteins (*see* Subheading 12.), or nonhomologous events in which the limited sequence identity is used in an alignment step prior to rejoining. One noncrossover gene conversion between *Alu* elements has been reported in what appears to be a *bona fide* recombination event *(71).* Because such conversions within *Alu* elements would not be expected to lead to gene mutations, they are likely to be underrepresented in tallies of *Alu-Alu* recombination events. At least a portion of events involving *Alu* elements occur by nonhomologous mechanisms, i.e., when single *Alu* elements become joined to unrelated sequences.

In addition to sequence divergence, relative location of *Alu* elements needs to be considered in evaluating the potential for *Alu-Alu* recombination in DSB repair. *Alu* elements can be located nearby each other, within a few kb or less, similar to the direct repeat recombination substrates described above. This suggests that nearby *Alu* elements (or other repetitive elements) may be used for recombination if the barrier arising from sequence divergence can be overcome. Alternatively, the large number of *Alu* elements found on other chromosomes or at greater distance on the same chromosome suggests recombination may be possible between more distantly located elements, as observed for interchromosomal recombination in ES cells (Subheading 8.2). In the mouse, recombination between diverged LINE-1 elements has been observed in experiments in which one element is on a transfected plasmid *(7,116).* More recently, a DSB in a chromosomal LINE element has been shown to be repaired by gene conversion with various endogenous LINE elements *(142a).*

9.2. Effect of Sequence Divergence on Recombination

In model systems examining the effect of sequence heterology, spontaneous recombination rates have been shown to be very dependent on the degree of identity of the recombining sequences, for both direct repeat chromosomal recombination *(150)* and gene targeting *(141).* The effect of sequence heterology on DSB-promoted recombination has been examined using the I-*Sce*I system *(35,140).* In DSB-promoted gene targeting in ES cells, increasing numbers of single bp polymorphisms were found to lead to progressively lower frequencies of recombination *(35).* The range in the amount of heterology was narrow, between 0.8 and 1.2%, with the decrease in recombination estimated to be between 2.5- and 6-fold, respectively. Because a 20-fold decrease in spon-

taneous gene targeting is observed for substrates with an estimated 0.6% divergence *(141),* spontaneous gene targeting may be more sensitive to sequence divergence. However, in these experiments the 0.6% heterology included small sequence insertions and deletions, which may have a greater effect on recombination than single bp polymorphisms. Interestingly, the barrier to recombination between the diverged sequences is relaxed when the mismatch repair gene *msh2* is mutated *(30),* consistent with results obtained in other organisms (*see* e.g., *113*).

In addition to the overall amount of heterology, the length of perfect homology is also important in spontaneous recombination. A decrease from 232 to 134 bp of uninterrupted homology decreased recombination 20-fold *(149).* There are indications that this is also true for DSB-promoted events, although less so *(35).* The position of polymorphisms relative to the DSB may also affect recombination, because a single polymorphism interrupting the homology adjacent to a DSB was shown to consistently lower the frequency of recombination *(35).*

The precise position of the DSB introduced by I-*Sce*I allows a determination of the extent of gene conversion from a DSB. Similar results have been obtained with direct repeats *(140)* and gene targeting *(35).* Gene-conversion tract lengths were found to be short, typically less than 100 bp, even when homology increased from 0.7 kb *(35)* to 1.4 kb *(140).* The short tract lengths imply that chromosome ends are protected from extensive degradation. In homolog recombination, in which homology extends the length of the chromosome, longer gene-conversion tracts were observed in some recombinants *(99).*

10. OTHER FACTORS THAT INFLUENCE RECOMBINATION

10.1. Transcription and DSB-Induced Recombination

Transcription has been shown both in yeast and mammalian cells to stimulate direct-repeat recombination *(100).* It has been postulated that transcription increases the number of initiating events, either directly by increasing the frequency of lesions or indirectly by increasing accessibility to recombination enzymes. To address this question, the effect of transcription on DSB-induced recombination was examined *(140).* No further stimulation of DSB-induced recombination was found when the recombination substrates were highly transcribed. In addition, the spectrum of gene-conversion events was found to be similar under conditions of low and high transcription *(140).* These results are consistent with a role for transcription in increasing the number of initiating events, rather than affecting later steps in the recombination pathway.

10.2. Hairpin Structures

Hairpin structures in chromosomes are susceptible to strand breakage and, therefore, are a potential source of genetic instability. These structures can form at the center of symmetry of perfect inverted repeats (palindromes), inverted repeats separated by spacers, or quasipalindromic sequences such as triplet repeats. Palindromes manifest a higher degree of instability than imperfect inverted repeats, presumably owing to more efficient hairpin extrusion and subsequent strand breakage. Single-strand breaks or DSBs can be introduced at hairpin tips by nicking enzymes which appear to be ubiquitous in mammalian cells *(76).* The Rad50 complex (*see* Subheading 12.3.), which has

been shown to introduce nicks into hairpins in vitro *(104),* has been proposed to be involved in this process in vivo *(1).* Strand breaks have also been presumed to arise at the base of hairpins. Because hairpins impede the progression of replication forks, breaks are likely to arise owing to stalled replication, as described in *E. coli* and yeast *(50,75).* In humans, quasipalindromic triplet repeats are very unstable presumably as a result of replication slippage at stalled replication forks *(2).*

In the mouse germline, instability of palindromes leading to both nonhomologous and homologous rearrangements is observed *(1,26).* Nonhomologous rearrangements are frequently deletions at the center of symmetry, consistent with hairpin-induced strand breaks that are nonhomologously rejoined. Because the deletions are not symmetrical, the palindrome is resolved into an imperfect inverted repeat that is genetically more stable than a perfect palindrome. Homologous recombination is also increased at palindromes, including gene conversions within the palindromic repeats and other types of recombination *(1).*

10.3. Transposable Elements

Transposable element excision in some organisms can be the source of DSBs that cause genetic instability. For example, P-element excision in *Drosophila (36)* and Tc 1 excision in *C. elegans (111)* result in DSBs that induce a high frequency of recombination between homologs. Although active endogenous transposons have not yet been identified in mammalian cells, transposable elements of the mariner family have been suggested to be responsible for creating a recombination hotspot that is responsible for some inherited neuropathies *(114).* Transposable elements in mammalian genomes are much less abundant than retrotransposable elements such as *Alu* repeats, and those that have been identified are transpositionally inactive owing to the accumulation of mutations *(72).* However, it has been possible to reconstruct an active vertebrate mariner transposon by eliminating inactivating mutations, raising the possibility that active elements exist *(61).*

11. OTHER OUTCOMES OF REPAIR OF INDUCED DSBs

11.1. Telomere Addition

The addition of new telomeres to the ends of broken chromosomes, termed chromosome healing, has been extensively studied in organisms such as *Tetrahymena.* This process has been shown to be dependent on telomerase *(156).* Telomerase, a reverse transcriptase-like enzyme, has an RNA cofactor that templates the addition of simple sequence repeats onto the ends of chromosomes *(157).* In addition to being required for proper DNA replication of chromosome ends, telomeres protect chromosomes from undergoing end-to-end fusions that lead to breakage/fusion/bridge cycles *(91)* by forming specialized structures, called t loops *(53).* A telomere binding protein, TRF2, is apparently critical for this structure because disruption of its binding can lead to end-to-end fusions of chromosomes in human cells *(145).*

To detect chromosome healing after DSB repair in mammalian cells, an I-*Sce*I site and a *TK* gene were integrated adjacent to a telomere in ES cells *(133).* Upon expression of I-*Sce*I, terminal deletions were identified by selecting *TK*$^{-}$ cell clones, analysis of which showed that telomeric repeats were added to the break site, in some cases

directly to the I-*Sce*I overhang. The number of repeats initially was less than in the parental cell line, but they increased after continued cell culture. Two different mechanisms of telomere healing are possible: the *de novo* synthesis of new telomeric repeats by telomerase, or the addition (or copying) of pre-existing telomeres. The contribution of these two mechanisms has not conclusively been determined, although results are consistent with *de novo* telomere synthesis *(133).*

11.2. Gene Amplification

An infrequent outcome of DSB repair is gene amplification. Breaks induced at fragile sites are known to trigger breakage/fusion/bridge cycles, presumably owing to the fusion of broken sister chromatids *(29).* After mitosis, the fused sister chromatids break asymmetrically, doubling the copy number of sequences near the fragile site in one of the daughter cells (*see* also *89*). Continued rounds of amplification lead to larger copy numbers. A hallmark of this type of amplification is loss of sequences distal to the break site.

Taking advantage of the insertion of an I-*Sce*I site distal to the dihydrofolate reductase gene *(DHFR)* in hamster cells, it has been possible to select amplification events as a result of I-*Sce*I-induced DSB repair *(109).* The structure of the amplification events after I-*Sce*I cleavage were compared with those that were induced at a fragile site located distal to the *DHFR* gene *(29).* As with the fragile site, amplifications induced with I-*Sce*I led to loss of sequences distal to the I-*Sce*I site. Because the I-*Sce*I site is closer to the *DHFR* gene than the fragile site, a larger portion of the distal region of the chromosome was lost with I-*Sce*I-induced amplification, confirming that they were initiated from the I-*Sce*I site. Thus, it appears that a DSB induced by I-*Sce*I is able to initiate the same sequence of events leading to *DHFR* amplification as a DSB occurring at a fragile site. This suggests that amplifications initiated at fragile sites reflect the high probability of breakage, rather than some other contribution to the amplification process *(109).*

12. ANALYSIS OF DSB REPAIR MUTANTS AND OVEREXPRESSION OF RECOMBINATION PROTEINS

IR-sensitive mammalian cell mutants have been identified that are defective in nonhomologous repair processes (Vol. 2. Chapter 16). The demonstration that homologous recombination is a major repair pathway implies that some IR-sensitive mutants may have defects in recombination proteins. This is indeed the case both for mutants obtained from targeted mutagenesis and from more standard mutant screens for IR-sensitive cell lines.

12.1. Rad51

Rad51 knockout mice display the most severe phenotype to date for disruption of a purported DSB repair gene. Rad51 is a RecA homolog *(98,129),* and has strand-transferase activity *(5)* which is expected to result in the formation of recombination intermediates (*see* Chapter 6). Disruption of the mouse *Rad51* gene leads to very early embryonic lethality *(82,144).* This contrasts with mouse mutants that are defective in nonhomologous repair process, which exhibit a late embryonic lethality *(42,46)* or are viable *(45,54,78,101,138).* Although cell lines cannot be established from the embryos,

short-term cultures of cells recovered from the dying *Rad51*$^{-/-}$ embryos are IR-sensitive and have sharply reduced chromosome numbers *(82).* These results point to a key role for Rad51 in the repair of endogenous and exogenous DNA damage.

Overexpression studies as well as mouse knockouts are consistent with a role for Rad51 in recombination. Human cell lines immortalized by SV40 T antigen have increased expression of Rad51 over control lines and a parallel increase in spontaneous intrachromosomal recombination, up to seven fold *(153).* Similarly, overexpression of hamster Rad51 in CHO cells increases intrachromosomal recombination by 20-fold *(147).*

12.2 Rad51-Related Proteins

Mammalian Rad51 shares approx 70% sequence identity with the yeast Rad51 protein. A number of other Rad51-related proteins have been identified in mammalian cells that share much less sequence identity (*see* Chapter 15). These proteins have been shown to interact with each other and with Rad51 in a number of pairwise combinations *(33,86)* possibly acting together in a complex *(128a).* Three of the Rad51-related proteins, Rad51B *(17,115),* Rad51C *(33),* and Rad51D *(110),* have been identified by database searches and although cell lines mutated for the genes have yet to be reported, mutation of at least two of the genes results in embryonic lethality *(110a,129a).* Two other Rad51-related proteins, XRCC2 and XRCC3, were identified as being defective in hamster cell mutants *(18,86).* The cell mutants, irs1 and irs1SF exhibit chromosomal instability, a weak IR sensitivity, and an extreme sensitivity to DNA crosslinking agents *(44,69).* These cell lines also have chromosome segregation defects *(52a).* The *XRCC2* mutation in irs1 cells is apparently a null allele *(86)* indicating that, in contrast to Rad51, the XRCC2 protein is not necessary for cell viability. DSB-promoted recombination has been examined recently in both the irs1 *(68b)* and irs1SF *(13a,108a)* cells lines and has been found to be significantly reduced in both, although not totally abolished. It is possible that these proteins act as accessory proteins to Rad51 function, similar to the Rad51 related proteins in yeast *(58,68,135),* rather than having Rad51-like strand transferase activity.

12.3. Other Homologs of Yeast Rad52 Epistasis Group Proteins

In yeast, Rad51 is member of the Rad52 epistasis group of proteins that is involved in recombinational repair of DSBs (Vol. 1, Chapter 16). Other members of this group, Rad54 and Rad52, have mammalian homologs that have been disrupted in mice. Disruption of *Rad54* results in IR sensitivity and decreased gene targeting in ES cells, although *Rad54*$^{-/-}$ mice are viable and fertile *(37).* A defect in sister chromatid recombination has also been reported recently *(33a).* Disruption of *Rad52* has an even milder effect than *Rad54,* with only slightly reduced gene targeting (30% decrease) and no sensitivity to IR *(119).* Overexpression of human Rad52 in monkey CV1 cells results in a two-fold increase in spontaneous direct repeat recombination, smaller than with Rad51 overexpression *(102).*

Two other mammalian homologs to Rad52 epistasis group members have been cloned, Rad50 *(31)* and Mre11 *(107).* These two proteins are complexed with a third protein called p95 or NBS1 *(16),* which is deficient in patients with Nijmegen breakage syndrome *(16,146),* one of the chromosome instability syndromes (*see* Subheading 12.5.). This complex in yeast has multiple functions, being involved in both meiotic and

mitotic homologous recombination and nonhomologous end-joining *(55).* It is expected that its role in mammalian cells will be similarly complex, although little information is currently available. (For more information about these proteins, *see* Chapter 7.)

12.4. Chromosome-Instability Syndromes

Cell lines derived from patients with chromosome-instability syndromes (Vol. 2 Chapter 19) provide an additional source of mutants in which to study recombination. Common to these syndromes is a high frequency of chromosome breakage and IR sensitivity. For one of these syndromes, ataxia telangiectasia (A-T), a high level of spontaneous intrachromosomal recombination has been observed *(92).* The hyper-recombination phenotype may be the result of a higher frequency of chromosome breaks or their longer persistence, with recombination mechanisms *per se* not being affected. Further analysis is necessary to establish this point. Homologous recombination has yet to be examined in cell lines from patients with the other syndromes. NHEJ is not affected in these syndromes, as extrapolated from proficiency in V(D)J recombination *(60).*

13. RECOMBINATION GENES AS CARETAKERS AGAINST TUMORIGENESIS

13.1. Mutator Phenotypes and Cancer

Tumor-suppressor genes can be broadly divided into two groups termed "gatekeepers" and "caretakers" *(73).* Gatekeepers are genes that regulate cell proliferation and cell death, and, thus, have a direct role in guarding a cell from becoming tumorigenic. Caretakers play an indirect role by maintaining genetic integrity. Several genetic "hits" apparently need to occur for a cell to become tumorigenic *(96),* yet low cellular mutation rates suggest that this should be nearly impossible. However, cells that have acquired a mutator phenotype, by definition, have higher mutation rates and thus can be expected to accumulate the necessary number of mutations *(87).* This is supported by mutations in caretaker genes in several familial cancer syndromes *(148),* including in genes involved in mismatch repair (colon cancer; *10*) and nucleotide excision repair (skin cancer; *12*).

The identification of homologous recombination as a major DNA repair pathway in mammalian cells implies that disruptions in the recombination machinery might lead to a mutator phenotype *(62).* Consistent with this, patients with chromosome-instability syndromes have increased frequencies of a variety of malignancies (Chapter 19, Vol. 2) although the role that recombination plays in the etiology of these malignancies has yet to be established. Several types of mutations may be predicted to occur when homologous recombination is altered, such as increased frequencies of deletions, insertions, and possibly translocations, owing to a greater reliance on NHEJ, and unequal sister chromatid exchanges or LOH, owing to altered outcomes of recombination events. The latter idea is particularly appealing owing to the observance of LOH in tumor cells *(19).*

13.2. Hereditary Breast-Cancer Syndromes

The most striking connection between recombination and tumorigenesis is in hereditary breast-cancer disease (*see* Chapter 10). Proteins encoded by the genes

associated with these cancers, BRCA1 and BRCA2, have been shown to interact with Rad51. The interaction between BRCA2 and Rad51 is direct, being detected both by two-hybrid assays *(94,127)* and co-immunoprecipitation *(20,21,90).* For BRCA1, the interaction between BRCA1 and Rad51 may be indirect, possibly mediated by BRCA2 *(20,126).*

Like the Rad51 knockouts, mouse knockouts of BRCA1 and BRCA2 show early embryonic lethality *(52,56,85,127,128,136).* When cells are recoverable from these mutants, they are sensitive to DNA damaging agents and exhibit chromosome abnormalities *(27,51,97, 103,128,154,155).* Taken together, these results strongly suggest that Rad51-mediated homologous recombination is disrupted in cells with mutated *BRCA1* or *BRCA2* genes. Direct evidence for a role for BRCA1 homologous recombination has recently been obtained *(99a).* It will be important to determine if and how recombination defects can lead to tumorigenesis. Loss of BRCA1 protein has also been associated with sporadic breast carcinomas *(152),* suggesting that disrupted recombination may be important in the pathogenesis of both sporadic and hereditary breast and ovarian cancers.

14. CONCLUSION

The last few years have witnessed a tremendous growth in our understanding of homologous recombination in mammalian cells. It is now clear that homologous recombination is a major DNA repair pathway in mammalian cells. Of the available templates for repair in the cell, sister chromatids are preferred although recombination can occur between two different chromosomes. The ability of DSBs to stimulate recombination suggests a role for homologous recombination in maintaining genetic integrity after exposure to DNA damaging agents. A number of the genes involved in recombination have been identified, either by homology with yeast genes involved in recombination, or as genes mutated in radiation-sensitive cell lines, and study of these genes will help elucidate mechanisms of recombination. Finally, a caretaker role appears likely for homologous repair proteins in preventing cells becoming tumorigenic. Further research in this area offers much promise in the future for understanding the maintenance of genomic integrity.

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10

BRCA1 and BRCA2 in DNA Repair and Genome Stability

Mark A. Brenneman

1. INTRODUCTION

Since their discovery, the two major breast-cancer susceptibility genes *BRCA1* and *BRCA2* have challenged clinicians and the basic research community to understand their biological roles, and how their loss contributes to tumorigenesis. Progress has been rapid, accelerating particularly over the last three years, and there is now a foundation of evidence on the functions of the BRCA1 and BRCA2 proteins at the cellular and molecular levels. Each protein has been associated with multiple functions (Table 1). Moreover, BRCA1 and BRCA2 show a striking degree of functional overlap, with roles for both proteins in DNA repair, cell-cycle checkpoints, control of centrosome function, and transcriptional regulation. Much work remains to be done in elucidating the molecular details of how BRCA1 and BRCA2 perform these roles, but it is clear even at this early stage that BRCA1 and BRCA2 are central to the preservation of genomic integrity in mammalian cells. This chapter will review experimental evidence for BRCA1 and BRCA2 function in DNA repair and genomic stability. Not covered here is the voluminous literature on the spectrum of *BRCA1* and *BRCA2* mutations associated with cancer, their population genetics, and their clinical correlates. An overview of this literature can be found in reference *(13).*

2. STRUCTURAL FEATURES AND EXPRESSION OF BRCA1 AND BRCA2

When first described, the predicted protein sequences of human BRCA1 and BRCA2 were conspicuous for two features; their size and their uniqueness. BRCA1, with 1863 amino acids, is a large protein, whereas BRCA2, at 3418 amino acids, is enormous. Early hopes that the sequences of BRCA1 and BRCA2 might shed some light on their functions were thwarted by an almost complete lack of sequence similarity to any other known proteins. Over time, however, some important structural features of each protein have emerged that can now be related to known or proposed functions (Fig. 1).

2.1. The BRCA1 Protein

The human *BRCA1* gene is located on chromosome 17 (17q21). It was originally described as comprising 24 exons *(98).* Exon 4 was later found to be a cloning artifact,

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Table 1
Functional Roles of BRCA1 and BRCA2

Function	BRCA1 (Ref.)	BRCA2 (Ref.)
Transcription-coupled repair	*(2,53)*	(*see* Subheading 5.)
Homologous recombinational repair	*(105)*	(*see* Subheading 6.)
G2/M checkpoint induction	*(46,77,166)*	*(30)*
Centrosome regulation	*(166)*	*(152)*
Transcriptional regulation	*(28,29,37,101,107)*	*(90,99,178)*
Chromatin remodeling	*(64,171)*	*(50,132)*
Ubiquitination/deubiquitination	*(47,66,85)*	

but the original numbering of exons has been retained *(13)*. The *BRCA1* gene gives rise to several distinct mRNAs owing to differential use of transcription start sites and splicing signals, including mRNAs with alternative first exons, a form that omits all of exons 9 and 10, and forms that omit most or all of exon 11, the large central exon that encodes over half of the BRCA1 protein *(86,142,156,160,165)*. The full-length human BRCA1 protein of 1873 amino acids (approx 220 kD) is usually the most abundant form, but smaller isoforms, including ones that correspond to exon 11-deletion splice variant mRNAs have been reported. Nuclear localization signals have been identified within the region encoded by exon 11 *(31,101,142,160)*.

The amino-terminus of BRCA1 contains the cysteine-rich RING motif, a zinc-binding domain that has been identified in a large number of proteins associated with regulatory functions (Fig. 1). RING motifs participate in protein-protein interactions associated with diverse cellular functions *(21)*, and the RING motif in BRCA1 evidently mediates heterodimer formation with BARD1. Another recently proposed general role for RING motifs is in modulating ubiquitination reactions. A number of RING finger proteins, including BRCA1, are able to facilitate the catalytic activity of ubiquitin-conjugating enzymes *(47,85)*, and a deubiquitinating enzyme, BAP1, specifically interacts with the RING motif of BRCA1 *(66)*.

The carboxy-terminus of BRCA1 contains a repeated motif of about 95 amino acids, known as the "BRCT" (BRCA1 carboxy-terminus) domain, versions of which have been identified in a large number of proteins from several eukaryotic species *(23,26)*. The shared attribute of these various proteins is that most have some relation to DNA repair and/or cell-cycle regulation. The BRCT domain appears to function generally in mediation of protein-protein interactions *(92,128,140,176)*, but has also been shown to bind DNA ends *(169)*. Roles ascribed specifically to the BRCT domain in BRCA1 include transcriptional activation or coactivation, chromatin remodeling, and activation of replication, and the mediation of interactions with several other proteins, including p53, RNA helicase A, the transcriptional co-repressor CtIP, and components of the histone deacetylase complex *(4,28,29,64,81,101,107,171,172)*. (Fig. 1).

BRCA1 undergoes changes in phosphorylation during the course of the cell cycle and in response to DNA damage. It becomes hyperphosphorylated late in G1 and in S phase, and undergoes dephosphorylation shortly after M phase *(121)*. G1/S hyperphosphorylation at one site, serine 1497, is evidently mediated by cyclin-dependent kinase 2 *(120)*. In S-phase cells exposed to DNA-damaging treatments, BRCA1 undergoes fur-

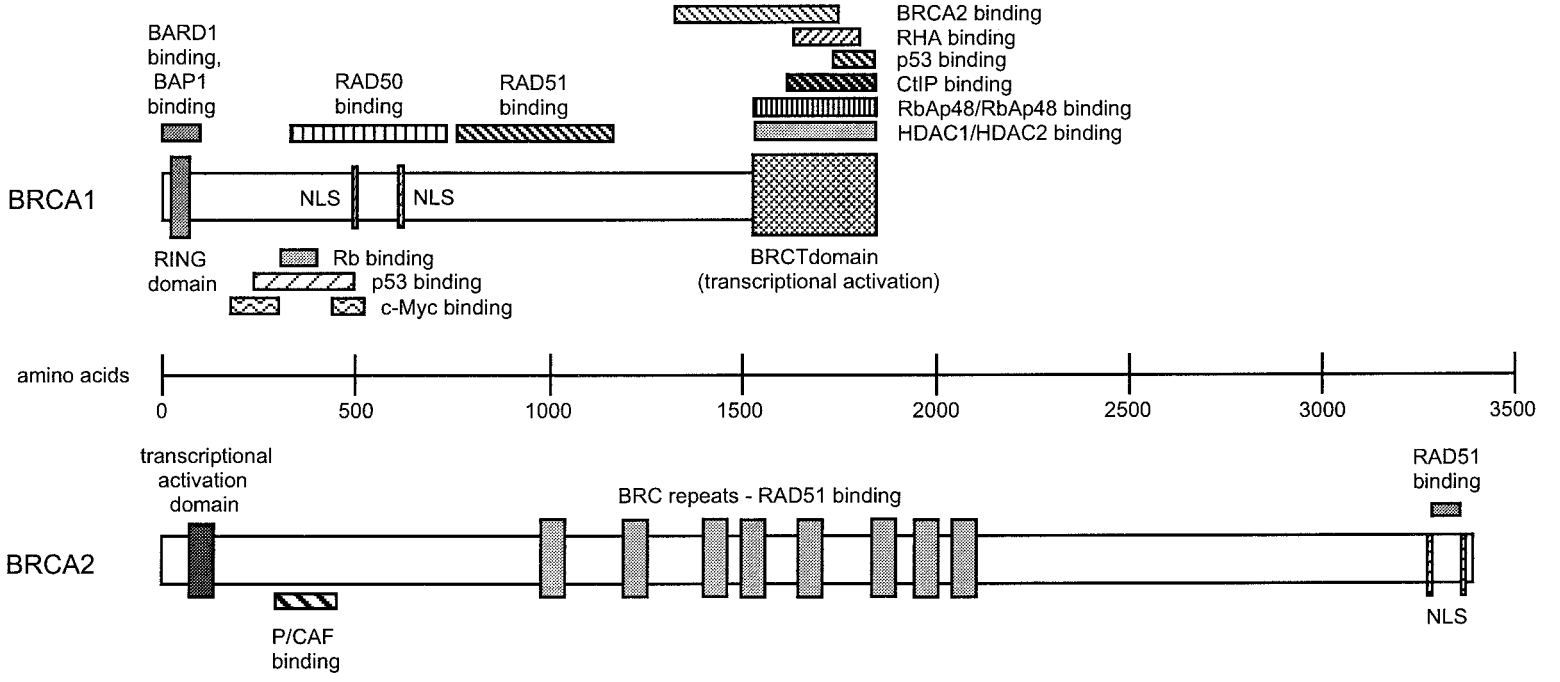


Fig. 1. Functional and protein interaction domains of BRCA1 and BRCA2. The positions of identified functional domains of BRCA1 and BRCA2 are indicated, together with regions that mediate specific protein interactions. This figure is not exhaustive; interactions have been demonstrated, but not yet mapped, for a number of proteins in addition to those shown here (*see* Table 2).

ther phosphorylation, which is accompanied by changes in subnuclear localization *(123).* The damage-induced hyperphosphorylation of BRCA1 is at least partly dependent on the checkpoint protein kinases ATM and CDS1*(41,80).*

The mouse *BRCA1* gene has also been cloned. Its predicted protein is shorter (1812 amino acids), and moderately conserved relative to human, with 58% amino acid identity and 73% similarity overall. The RING domain is perfectly conserved, however, and the BRCT domain is also highly conserved (83% identity), underscoring the probable importance of these domains in the critical functions of BRCA1 *(3,76,127).*

2.2. The BRCA2 Protein

The human *BRCA2* gene resides on chromosome 13 (13q12-q13). Its major transcript comprises 27 exons, but there is a reported splice variant that deletes exon 3 *(178).* Similarly to BRCA1, about half of BRCA2's length is encoded by a large central exon (exon 11). Despite this resemblance in exon structure, BRCA1 and BRCA2 have no significant sequence similarity to each other. BRCA2, like BRCA1, is moderately conserved across mammalian species. The mouse and rat BRCA2 proteins (3328 amino acids) are 59% and 58% identical (73 and 72% similar), respectively, to the human protein *(95,125).*

Exon 3 of human *BRCA2* encodes a region that bears some sequence homology to the transcriptional transactivation domain of c-Jun, and can exert transcriptional activation in yeast or mammalian cells when coupled to DNA-binding domains *(99).* A mutation occurring within this region in some familial breast cancers (tyrosine to cystine at codon 42) sharply reduces transcriptional activation. A splice site mutation that produces an in-frame deletion of exon 3 has been identified in a subset of endrometrial carcinomas that also show molecular evidence of replication and repair errors *(73).* These observations suggest that transcriptional activation may be important in tumor suppression.

Beyond its size, exon 11 of *BRCA2* is remarkable for encoding eight repeats of a loose consensus sequence, variously defined as from 26 to about 80 amino acids long, termed the BRC motif *(19,22).* These are unique to BRCA2, and not to be confused with the BRCT domain of BRCA1. Although exon 11 is poorly conserved overall, the BRC repeats are comparatively well-conserved across mammalian species *(19).* The BRC repeats are now known to mediate interaction between BRCA2 and the RAD51 repair and recombination protein (Subheading 4.2.) and their conservation argues for the importance of this interaction in BRCA2 function. Exon 11 contains other small regions, in addition to the BRC repeats, that are relatively well-conserved, most strikingly a stretch of 25 amino acids between the first and second BRC repeats that is identical in human, green monkey, dog, hamster, and mouse *(19).* No function has been proposed for this motif as yet.

The extreme carboxy terminus of BRCA2, encoded by exon 27, is also notable for two important features (Fig. 1). First, an additional RAD51 interaction domain, distinct from the BRC repeats, has been demonstrated in mouse BRCA2 *(100,126).* This domain is comparatively well-conserved between human and mouse, showing 72% identity. Second, the two functional nuclear localization signals that have been identified in human BRCA2 (amino acids 3263–3269 and 3381–3385) fall within this small region *(135).* Only the first of these is conserved in mouse BRCA2.

2.3. Expression Patterns of BRCA1 and BRCA2

Both BRCA1 and BRCA2 are predominantly nuclear proteins in normal cells *(16,31,101,121,123,142,143,156,160).* Moreover, failure of nuclear localization has been proposed in regard to both proteins as a cause of abnormal function in carcinogenesis. Wild-type BRCA1, although localized to the nucleus in normal cells and in nonbreast tumor cell lines, has been found to be cytoplasmic when expressed in breast-cancer cell lines *(31,36).* The reason for this apparent mislocation specifically in breast-cancer cells is unknown, and its significance has been controversial (reviewed in *38).* For BRCA2, the great majority of the mutations identified in cancers are small sequence changes predicted to result in truncated proteins. Even the most 3′ of known cancer-associated truncating mutations, at codon 3195, results in loss of the nuclear localization signals. It has been proposed that most cancer-associated alleles of BRCA2 are nonfunctional at least in part because the truncated proteins they encode fail to reach the nucleus *(135).* As detailed in later sections, the functions of BRCA1 and BRCA2 relating to DNA repair and genomic stability are expected to operate largely if not entirely in the nucleus.

Expression of both BRCA1 and BRCA2 is regulated during the cell cycle. For both BRCA1 and BRCA2, levels of mRNA and protein rise at the end of G1, prior to the onset of replication, and peak during S and G2 phases *(16,56,118,136,154,155).* For both BRCA1 and BRCA2, mRNA is detectable at low levels during early G1 phase in actively cycling cell populations, but is not detected in quiescent cells *(158).* The cell-cycle expression profiles of BRCA1 and BRCA2, and the absence of expression in non-dividing cells were early indications that both proteins are involved in cell proliferation.

The tissue distributions of BRCA1 and BRCA2 expression give further evidence of involvement in cell proliferation. In mouse, both genes are expressed at high levels during embryonic development, whereas in adult animals, expression of BRCA1 and BRCA2 is highest in rapidly proliferating tissues, including ovary, testis, and thymus, and in breast during puberty and pregnancy *(40,90,117,125).* The tissue localization of the two proteins is generally concordant with proliferating cell nuclear antigen (PCNA) *(20).* Expression is particularly high in the meiotic cells of mouse testis *(20,174),* which first suggested a role in meiosis, and hence in homologous recombination.

3. CELLULAR PHENOTYPES OF BRCA1 AND BRCA2 MUTANTS

Many insights into the functions of BRCA1 and BRCA2 have been gained through the creation of mice bearing targeted mutations of the *BRCA1* or *BRCA2* genes, and from the identification of human tumor cell lines that express only mutant BRCA1 or BRCA2. Analysis of these mutants produced much of the evidence implicating BRCA1 and BRCA2 as proteins involved in DNA repair and essential to genomic stability. Defects in cell proliferation and hypersensitivity to certain forms of DNA damage in *BRCA1* and *BRCA2* mutants were among the first indications of these roles, setting the stage for discovery of specific defects in transcription-coupled repair (TCR) and homologous recombinational repair (HRR), cell-cycle checkpoints, and chromosomal stability.

3.1. Embryonic Lethality and Defects in Cell Proliferation

In mouse, BRCA1 is required for embryonic development. Targeted mutations that truncate, or delete even a small part of the protein, almost always result in the death of

homozygous embryos *(54,60,83,87,129).* The time of developmental arrest is somewhat variable, but in all instances occurs before embryonic day 13, and usually before day 8 or 9. The only exceptions to early death of *BRCA1*$^{-/-}$ embryos are three double-mutant *BRCA1*$^{-/-}$ *p53*$^{-/-}$ mice reported by Cressman et al *(43,44).* The development to term and brief survival of these exceptional animals were proposed to reflect additional, unknown genetic alterations. The more general finding has been that developmental arrest and death of *BRCA1*$^{-/-}$ embryos is only briefly delayed when *BRCA1* mutations are bred into *p53*- or *p21*-null backgrounds *(43,44,59,87,129).*

Cellular proliferation is reduced in *BRCA1*$^{-/-}$ embryos in vivo, and when *BRCA1*$^{-/-}$ blastocysts are explanted their growth is grossly impaired *(60,83,129).* This seems not to be mediated by increased apoptosis *(60).* Growth impairment may result in part from the dramatic overexpression of p21 seen in *BRCA1*$^{-/-}$ embryos *(60).* However, the failure of either *p21*- or *p53*-null mutations to rescue completely embryonic development or blastocyst growth in culture suggests that impaired cell proliferation is owing to something more than the activation of cell-cycle checkpoints *(43,44,59,87,129).* Mouse *BRCA1*$^{-/-}$ cells have generally failed to proliferate in vitro (*see,* for example, *129),* with two notable exceptions: *BRCA1*$^{-/-}$ mouse embryonal (ES) cells and primary fibroblasts from *BRCA1*$^{-/-}$ p53$^{-/-}$ mice derived in the lab of B. H. Koller *(43,53,54).* Early passage *BRCA1*$^{-/-}$ *p53*$^{-/-}$ primary fibroblasts grew more slowly and reached lower saturation densities than control *p53*$^{-/-}$ fibroblasts. While they traversed the cell cycle with kinetics similar to *p53*$^{-/-}$ controls, the *BRCA1*$^{-/-}$ *p53*$^{-/-}$ fibroblasts showed a higher rate of cell death such that the population growth rate was lower.

The influence of BRCA1 on cell proliferation has also been examined in human tumor cell lines, but these experiments are somewhat difficult to reconcile with observations in mouse. In human cells, BRCA1 can inhibit growth. Antisense inhibition of BRCA1 expression accelerates the growth of normal and malignant breast epithelial cells, but not of nonmammary epithelial cells *(145).* Overexpression of wild-type BRCA1 slows the growth of breast and ovarian cancer cell lines, but not cell lines from other tumor types *(62).* In particular, the growth of the *BRCA1*-mutant human breast-cancer line, HCC1937, is inhibited after stable transfection with full-length, wild-type BRCA1 *(2).* HCC1937 carries only the 5283insC frameshift allele of *BRCA1,* encoding a protein that lacks the BRCT domain *(149).* The discrepancy between a requirement for BRCA1 for mouse cell proliferation during development, and the inhibition of growth in human mammary or ovarian cell lines, may be more apparent than real. The growth defect in embryonic mouse cells may reflect a general requirement for BRCA1 in repair processes associated with DNA replication and in chromosome segregation. The inhibition of growth by BRCA1 in human breast and ovarian tumor lines may result from a more specialized growth-regulating function in the hormonal milieu of these cell types.

BRCA2, similarly to *BRCA1,* is essential for embryonic development in the mouse. Mutations that delete the large central exon 11 of *BRCA2,* or terminate translation 5′ to exon 11, result in embryonic death by day 8 or 9 *(87,126,138).* However, mutations that leave exon 11 intact, or that truncate within exon 11, allow embryonic development to term *(39,49,103,109).* These results strongly suggest that the ability of BRCA2 protein to interact productively with RAD51 via the BRC repeats is a critical determinant of embryonic viability (*see* Subheading 4). Mutations that delete all of the BRC repeats

fail to support development, whereas mutations that preserve some or all of the BRC repeats permit embryonic survival, though not without phenotypic consequence.

The ability to create sublethal mutations of *BRCA2* in mouse has made the derivation of *BRCA2*-mutant cells a good deal easier than for *BRCA1*. Primary mouse embryonic fibroblasts (MEF) bearing sublethal truncations of BRCA2 have consistently shown proliferative defects in culture. This is true for two different truncations within exon 11*(39,109),* and also for a milder truncation that removes only the extreme carboxy terminus of BRCA2 encoded by exon 27 *(24,103).* Their growth is markedly slower than wild-type controls, and cellular senescence occurs earlier; typically after only a few passages. Growth arrest is accompanied by high levels of p53 and p21 expression *(39,109).* Immortalized MEF were derived from primary MEF bearing the exon 27 truncation, though only with difficulty. These cells have overcome the block to proliferation seen in primary cells, perhaps through acquisition of other mutations, but still show reduced cloning efficiency relative to immortalized wild-type MEF *(24,103).*

A human *BRCA2*-mutant cell line also exists. The long-established human pancreatic-cancer cell line Capan-1 expresses BRCA2 truncated within the 3′ portion of exon 11 *(1,35,52).* In this way Capan-1 is similar to the targeted mutation in mouse *BRCA2* generated by Ashworth and coworkers *(39,152).* Though immortal, Capan-1 cells grow very slowly in culture and have extremely low cloning efficiency. Because Capan-1 is an aneuploid tumor line and presumably carries numerous mutations, its poor growth characteristics may not be owing (or entirely owing) to loss of BRCA2 function.

3.2. Sensitivity to DNA Damaging Agents

Cells mutated for *BRCA1* show hypersensitivity to ionizing radiation (IR), which induces DNA double-strand breaks (DSBs) and oxidative damage, and to hydrogen peroxide, which produces oxidative damage. Survival of $BRCA1^{-/-}$ mouse ES cells is reduced by about fivefold relative to $BRCA1^{+/+}$ cells after exposure to 8 Gy of γ-radiation, and by about threefold after treatment with 8 m*M* hydrogen peroxide *(53).* Primary fibroblasts from $BRCA1^{-/-}p53^{-/-}$ mice show similar hypersensitivity to IR and to hydrogen peroxide, and a very mild hypersensitivity to ultraviolet (UV) light *(43).* The human *BRCA1*-mutant cell line HCC1937 is also hypersensitive to IR, but much more so than the *BRCA1*-mutant mouse cells, with survival after exposure to 6 Gy of γ-radiation reduced more than 100-fold relative to $BRCA1^{+/+}$ human cells. Although HCC1937 is a tumor line and known to carry other mutations *(149),* its extreme hypersensitivity to IR appears to result mainly from *BRCA1* deficiency, because it can be largely relieved by expression of modified BRCA1 (partial peptides that do not exert the growth-suppressing effect of full-length human BRCA1) *(2).* BRCA1 may also be required for resistance to DNA interstrand crosslinks. Subclones of breast- and ovarian-cancer cell lines with spontaneously acquired resistance to the DNA crosslinking drug cisplatin show increased expression of BRCA1 relative to nonresistant clones *(65).* Antisense inhibition of BRCA1 expression in cisplatin-resistant cells resulted in loss of resistance correlated with reduced repair of crosslinks and increased apoptosis.

BRCA2-mutant cells are similarly hypersensitivity to IR. In early mouse embryos homozygous for a severely truncating (lethal) *BRCA2* mutation, the inner cell mass could be completely ablated by 4 Gy of γ-radiation, an exposure that had only slight effect on wild-type embryos *(126).* Mouse embryonic cells with less severe truncations

of BRCA2 also show exaggerated sensitivity to IR *(103,109),* and in one instance have been shown to repair the DSBs induced by IR less efficiently than wild-type cells *(39).* Capan-1 cells show extreme sensitivity to IR and to chemotherapeutic drugs that induce DSBs, and repairs such breaks less efficiently than human tumor lines not mutated for BRCA2 *(1).*

The observed sensitivities of *BRCA2*-mutant cells to DNA-damaging agents other than IR are somewhat confusing. In mouse cells, one group has observed hypersensitivity to UV light and to the methylating agent methyl methanesulfonate (MMS), but not to the crosslinking agent mitomycin C (MMC) *(109).* A second group found no hypersensitivity to UV, but marked hypersensitivity to MMC *(24,103).* The apparent discrepancies probably derive from differences in experimental methods and from the two very different BRCA2 mutants examined. UV hypersensitivity was seen in primary MEF homozygous for a mutation that truncates the BRCA2 after the third BRC motif *(109).* Absence of UV hypersensitivity was seen in ES cells bearing a milder truncation that deletes only the extreme carboxy terminal domain encoded by exon 27, and leaves all eight BRC repeats intact *(103).* Immortalized MEF bearing the same exon 27 deletion are hypersensitive to MMC when assayed by colony formation *(24).* A colony-formation assay was not possible for the primary MEF tested previously *(109),* and the endpoint used (trypan blue exclusion 48 after exposure) might not have detected mitotic arrest owing to unrepaired DNA crosslinks. Capan-1 cells are also hypersensitive to MMS *(35).* Wild-type monkey cells expressing a fragment of BRCA2 that exerts dominant-negative effects are hypersensitive to cisplatin and IR *(173).*

The hypersensitivity of BRCA1- and BRCA2-mutant cells to IR points to a defect in DSB repair. The association of both proteins with RAD51 (discussed in Subheading 4.) implies that homologous recombination is the affected repair pathway. Increased sensitivity of BRCA1- and BRCA2-deficient cells to cisplatin or MMC is also consistent with this idea, because interstrand crosslinks are thought to be repaired by homologous recombination *(48).* Roles in HRR has now been demonstrated for both BRCA1 and BCRA2 (Subheading 6.).

4. INTERACTIONS OF BRCA1 AND BRCA2 WITH DNA REPAIR PROTEINS, AND SUBNUCLEAR LOCALIZATION IN RESPONSE TO DNA DAMAGE

BRCA1 and BRCA2 participate in specific interactions with a large and growing list of other proteins, as summarized in Table 2. Most of these interactions can be related, directly or indirectly, to some aspect of cellular response to DNA damage.

4.1. BRCA1 and BARD1

BRCA1 interacts with BARD1 (BRCA1-Associated RING Domain), to which it is structurally related *(163).* Human BARD1 is a 777 amino acid protein that, like BRCA1, contains a zinc-binding RING motif near its N-terminus and a bipartite BRCT domain near its carboxy-terminal end. Unlike BRCA1, BARD1 also contains three centrally positioned ankyrin repeats. BARD1 and BRCA1 interact in vitro and in vivo, and it appears that their RING domains are necessary (though not sufficient) for this interaction *(97)* (Fig. 1). The interaction is likely to be important in BRCA1 tumor suppres-

Table 2
Proteins Interacting with BRCA1 and BRCA2

	Protein		References
BRCA1:	BARD1	BRCA1-associated ring domain	*(163)*
	BRCA2		*(33,34)*
	RAD51		*(34,124)*
	RAD50		*(159,177)*
	MRE11		*(159,177)*
	NBS1	Nijmegen breakage syndrome	*(159,177)*
	BLM	Bloom's syndrome – mutated	*(159)*
	RPC	Replication protein C	*(159)*
	ATM	Ataxia Telangiectasia – mutated	*(159)*
	MSH2	MutS homolog 2	*(159)*
	MSH6	MutS homolog 6	*(159)*
	MLH1	MutL homolog 1	*(159)*
	p53		*(28,175)*
	BAP1		*(66)*
	Rb	Retinoblastoma protein	*(7,171)*
	RbAp46/48	Rb binding proteins	*(171)*
	HDAC1/2	Histone deacetylase catalytic subunits	*(171)*
	RHA	RNA helicase A	*(4)*
	γ tubulin		*(63)*
	CtIP	CtBP-interacting protein	*(81,161,172)*
	c-Myc		*(157)*
BRCA2:	BRCA1		*(33,34)*
	RAD51		*(69,100,126,162)*
	p53		*(90)*
	P/CAF	p300/CBP-associated factor	*(50)*

sion. BRCA1 missense mutations within the RING domain that disrupt the BARD1 interaction correlate with breast-cancer susceptibility. Moreover, missense mutations in BARD1 itself, with accompanying loss of heterozygosity, have been found in a small number of breast and uterine cancers *(141).* BARD1, like BRCA1, is a nuclear protein, and its tissue distribution is similar to that of BRCA1 *(9).* Though expression levels of BARD1 are essentially constant over the cell cycle, BARD1 exhibits an S-phase-specific colocalization with BRCA1 within nuclear foci *(67),* and a similar pattern of subnuclear relocalization after DNA damage *(123)* (Subheading 4.2.).

4.2. Interactions of BRCA1 and BRCA2 with RAD51

Early clues that BRCA1 and BRCA2 might function in DNA repair and/or recombination came from findings that both physically associate with the RAD51 (Fig. 1.), that their expression patterns mirror those of RAD51, and that they co-localize with RAD51 in the nuclei of mitotic and meiotic cells. The significance of these findings stems from the central importance of RAD51 itself in homologous recombination and DNA repair. It is likely that the interactions of BRCA1 and BRCA2 with RAD51 lie at the heart of their critical functions in guarding the integrity of the genome and suppressing neoplastic transformation. To appreciate this requires some background on RAD51.

In the yeast *Saccharomyces cerevisiae,* Rad51p is required for the repair of DSBs, and for homologous recombination in both mitotic and meiotic cells. Yeast *rad51* mutants are extremely sensitive to IR and to drugs that induce DSBs or crosslinks, such as MMC *(51,102,115).* Rad51p has the ability to form nucleoprotein filaments with DNA, and to pair or transfer complementary DNA strands *(14),* much like *Escherichia coli* RecA, the prototypical recombinational strand transferase to which it is evolutionarily related.

Vertebrate homologs of yeast *RAD51* were identified on the basis of DNA sequence similarity *(15,17,104,130).* Like yeast Rad51p, mammalian RAD51 forms nucleoprotein filaments with DNA in vitro, has a DNA-dependent ATPase activity *(15),* and has strand-transfer activity in vitro *(10,11).* Expression of the mouse and human *RAD51* genes is cell-cycle dependent, with transcript present from late G1 phase to M phase *(32,168).* From late G1 through the end of G2 phase, RAD51 is localized to the nucleus *(168).* Antibody staining of mouse tissue sections shows that RAD51 is most highly expressed in actively proliferating tissues and in germ-line cells undergoing meiosis *(168).* In meiotic cells, immunofluorescence reveals that mammalian RAD51 associates with synaptonemal complexes *(8,57,114).* RAD51 becomes localized in nuclear foci that appear in premeiotic S phase and persist into metaphase I, suggesting that RAD51 has a role in the initial pairing of homologous chromosomes as well as in subsequent crossing-over. In somatic cells, RAD51 can be detected in discrete nuclear foci or "dots" during S phase and, after exposure to IR or other DNA damaging agents, reaggregates into larger, more irregularly distributed foci *(57,124,139).* These latter nuclear foci coincide with regions of single-stranded DNA, the substrate for formation of RAD51 nucleoprotein filaments, and are interpreted as sites at which recombinational repair complexes have formed *(116).*

The functions of RAD51 are essential in vertebrate cells, not only for repair of exogenous DNA damage but for cell growth and genomic stability. In mouse, knockout of *RAD51* results in early embryonic lethality *(82,151). RAD51*$^{-/-}$ embryos arrest before day six of development, apparently owing to a generalized failure of cell proliferation. The cells of very early *RAD51*$^{-/-}$ embryos are extremely sensitive to IR and, even in the absence of exogenous DNA damage, faltering cell proliferation is accompanied by chromosome loss and apoptotic cell death *(82).* A conditional *RAD51* knockout has been created in DT40 chicken lymphoblastoid cells. These cells grow normally as long as *RAD51* is expressed, but shutdown of *RAD51* expression results in cell-cycle arrest in G2/M phase, with numerous chromosome breaks, followed by cell death *(134).* The functions of RAD51 and the role of HRR in maintaining genomic integrity are more fully reviewed elsewhere *(12,18,131,144; see* also Chapter 9).

Association of BRCA1 with RAD51, in both mitotic and meiotic cells, was initially proposed on the basis of immunofluorescence and co-immunoprecipitation studies *(124).* Fluorescent antibodies to human BRCA1 localized it to discrete nuclear foci during S phase of the mitotic cell cycle. RAD51 similarly occupies discrete nuclear foci during S phase, which significantly (though not completely) overlap BRCA1 foci. Immunoprecipitates from human cell extracts made with antibodies to BRCA1 also contained RAD51, though only a small fraction of total cellular RAD51 was precipitated. Curiously, in the converse experiments, endogenous BRCA1 was not detected in immunoprecipitates made with antibodies to RAD51. However, it was possible to

detect small amounts of exogenously expressed, epitope-tagged BRCA1 in anti-RAD51 immunoprecipitates. In vitro, a peptide corresponding to amino acids 758–1064 of BRCA1 specifically, though weakly, co-immunoprecipitated RAD51. By immunofluorescence, BRCA1 and RAD51 colocalize on synapsing meiotic chromosomes in primary spermatocytes *(124).* Both proteins associate preferentially with the nonsynapsed (axial) portions of developing synaptonemal complexes.

The physiological significance of association between BRCA1 and RAD51 is supported by further studies showing that BRCA1 and RAD51 (and BARD1) disperse from their characteristic S-phase nuclear foci after cells are treated with hydroxyurea (to induce arrest of DNA replication) or DNA-damaging agents (UV, γ radiation, or MMC), and relocalize to regions positive for PCNA *(123).* The latter are presumed to represent sites of DNA replication. The redeployment of BRCA1 to PCNA-positive regions in UV-treated cells is accompanied by a dose-dependent increase in phosphorylation of BRCA1 *(123).* Similar dispersal of BRCA1 nuclear foci is seen in cells subjected to heat shock or herpes virus infection, and in cells infected with adenovirus, BRCA1 is recruited to regions of viral transcription and replication *(94).* It is not yet clear whether the interaction between BRCA1 and RAD51 is direct or indirect. Some aspects of the data suggest that it may be an indirect or perhaps very dynamic interaction, notably the small fraction of total nuclear RAD51 detected in anti-BRCA1 precipitates and the absence of BRCA1 in anti-RAD51 precipitates. It has been proposed that the interaction between BRCA1 and RAD51 might be mediated by a third protein. BRCA2 is a likely candidate, because of its interactions with both BRCA1 and RAD51.

The initial (and somewhat controversial) evidence for interaction between BRCA1 and RAD51 was followed swiftly by several independent demonstrations of physical association between BRCA2 and RAD51. Sharan et al. *(126)* used a yeast two-hybrid screen to detect an interaction between BRCA2 and RAD51 proteins from mouse, and confirmed the interaction in a mammalian two-hybrid system using mouse 3T3 cells. The interaction domains were mapped by deletional analysis to a highly conserved N-terminal region of RAD51 (amino acids 1–43) and a minimal domain comprising 36 amino acids (3196–3232) near the extreme C-terminus of BRCA2 that is also highly conserved (>90% identical to human BRCA2) (Fig. 1). Interaction between RAD51 and the C-terminus of mouse BRCA2 was independently discovered by Mizuta et al. *(100)* in a yeast two-hybrid screen, and confirmed in vitro using an affinity pull-down (GST fusion) assay.

However, it quickly became evident that the BRCA2 interaction with RAD51 involves additional domains. Analysis of human BRCA2 by yeast two-hybrid and affinity pull-down assays identified RAD51-binding domains within the central region encoded by exon 11 *(35,69,162)* and mapped them to the conserved BRC repeats (Fig. 1). Six of the eight BRC repeats, all except repeats 5 and 6, were able to function independently as RAD51-binding domains in the yeast two-hybrid system *(162).* When examined in vitro by affinity pull-down, it was found that all of the BRC repeats, including repeats 5 and 6, were able to independently bind RAD51 *(162).* Yeast two-hybrid analysis was further used to define the minimal region of RAD51 required for interaction with the BRC repeats of human BRCA2. This analysis, in contrast to the previous results for mouse *(126),* revealed that a C-terminal region of human RAD51 (residues 98–339) was required for interaction, and that the N-terminus was not *(69,162).* It appears that

the interaction between the N-terminus of RAD51 and the extreme C-terminal region of mouse BRCA2 may be wholly different from the interactions involving BRC repeats. The extreme C-terminus of human BRCA2 can interact with RAD51 in yeast two-hybrid experiments, but this interaction has not been mapped to a specific part of RAD51 (Z. Shen, personal comunication). BRCA2-RAD51 interaction has been confirmed in vivo by showing that antibodies specific to either protein can readily coimmunoprecipitate the other from cellular lysates *(35,89).*

BRCA1 and BRCA2 associate with each other as well as with RAD51. Antibodies raised against several different epitopes of BRCA1 can co-immunoprecipitate BRCA1 and BRCA2 *(33).* In reciprocal experiments, two different antibodies raised against epitopes in BRCA2 coimmunoprecipitated BRCA2, BRCA1, and RAD51. The part of BRCA1 that mediates interaction with BRCA2 maps to a carboxy-terminal region distal to amino acid 1313 (Fig. 1), but evidently does not include the extreme carboxy terminus, because the mutant BRCA1 in HCC1937 cells, which is truncated after amino acid 1755, can co-immunoprecipitate with BRCA2 *(33).* The part of BRCA2 that participates in association with BRCA1 has not been mapped, but appears to lie within the amino-terminal half of BRCA2, since the mutant protein in Capan-1 cells, truncated after amino acid 1981, co-immunoprecipiates with BRCA1 (and RAD51) *(33).* Like BRCA1 and RAD51, BRCA2 associates with meiotic chromosomes in spermatocytes, localizing preferentially to the nonsynapsed regions of developing synaptonemal complexes *(33).* In mitotic cells, BRCA2 localizes in discrete nuclear foci in S-phase cells, as seen for BRCA1, BARD1, and RAD51, and similarly relocalizes after treatment of cells with hydroxyurea to a distribution that overlaps PCNA foci *(33).* The formation of DNA damage-induced RAD51 nuclear foci may be dependent on BRCA2. Radiation-induced RAD51 foci are sharply reduced in Capan-1 cells, despite the fact that the truncated BRCA2 these cells express is able to bind RAD51 *(173).* Overexpression in wild-type cells of a single BRC repeat from BRCA2 can suppress the induction of RAD51 foci, and increase cellular sensitivity to DNA damage *(30,173).* Formation of RAD51 foci is probably not dependent on BRCA1, because it is unimpaired in HCC1937 cells *(177).*

The physical interactions of BRCA1, BARD1, BRCA2, and RAD51, together with their patterns of subnuclear colocalization and relocalization, suggest strongly that these proteins function together in the response of mitotic cells to DNA damage, and in meiotic recombination. It appears that BRCA1, BARD1, and BRCA2 participate in a protein complex with RAD51, that this complex is assembled specifically during S-phase of the cell cycle, and that it relocates to (or reassembles at) sites of damage in actively replicating DNA.

4.3. Interactions of BRCA1 with the RAD50-MRE11-NBS1 Complex

BRCA1 physically interacts and co-localizes in the nucleus of human cells with the RAD50-MRE11-NBS1 protein complex *(159,177).* This interaction is of great interest because of the central involvement of RAD50-MRE11-NBS1 in DNA damage sensing and repair. *RAD50* and *MRE11,* like *RAD51,* were originally identified in the yeast *S. cerevisiae* as members of the *RAD52* epistasis group, required for wild-type resistance to IR and for genetic recombination in mitotic and meiotic cells. In yeast, Rad50p and Mre11p function in complex with a third protein, Xrs2. Mutations in the *RAD50, MRE11,*

or *XRS2* genes are associated with a sharp reduction in the efficiency of nonhomologous repair of DSBs, with pleiotropic effects (usually reduced frequency) on various classes of homologous recombination events, and with shortening of telomeres. On biochemical and genetic evidence, the RAD50-MRE11-XRS2 complex has been implicated in diverse functions, including: the creation and processing of DSBs required to initiate meiotic recombination; nucleolytic resection of DNA ends in mitotic cells in preparation for HRR; nucleolytic processing and/or spatial juxtaposition of DNA ends for nonhomologous end-joining (NHEJ); and the sensing of DNA damage in connection with induction of the G2/M cell-cycle checkpoint (reviewed in *58).*

In mammals, RAD50 and MRE11 function in complex with NBS1, which is not a sequence homolog of yeast Xrs2p but may be a functional homolog. Functions of MRE11 and RAD50 have been difficult to analyze in mammals; knockout of either gene in mouse results in early embryonic lethality *(88,164).* A conditional knockout of MRE11 has been made in DT40 chicken lymphoblastoid cells *(167).* Shutdown of MRE11 expression results in cell-cycle arrest and chromosome breakage followed by cell death, though not quite so rapidly as shutdown of RAD51 *(134).* The *NBS1* gene is the affected locus in Nijmegen Breakage Syndrome (NBS) *(27,93,153).* Clinically, NBS is associated with retarded growth, immune deficiency, predisposition to cancer, and exaggerated reaction to radiation therapy. Cells from NBS patients exhibit frequent chromosome breakage and rearrangements, hypersensitivity to IR and other DNA damaging agents, and checkpoint abnormalities *(5,6,74,111,113,137,170).* RAD50-MRE11-NBS1 has DNA-unwinding and nucleolytic activities in vitro *(110,150).* In vivo, RAD50-MRE11-NBS1 relocalizes into discrete nuclear foci after treatments that induce DSBs *(91,106).* These foci are reminiscent of, but apparently distinct from, the damage-induced foci formed by RAD51. For a full review of RAD50-MRE11-NBS1, *see* Chapter 7.

BRCA1 is evidently involved in the DNA damage response functions of RAD50-MRE11-NBS1. Antibodies to BRCA1 co-immunoprecipitate RAD50 from human cell extracts and vice versa *(159,177).* As seen for RAD51, only a fraction of total cellular RAD50 coprecipitates with BRCA1. Using synchronized cell populations, the interaction was shown to be cell cycle-specific, with association between RAD50 and BRCA1 appearing during S phase and peaking during G2. In vitro affinity pull-down experiments and a yeast two-hybrid system were used to map the part of BRCA1 required for interaction with RAD50 to a central region (amino acids 341 to 748) that does not overlap the N-terminal RING motif or the C-terminal BRCT domain *(177)* (Fig. 1). The BRCA1-interaction domain of RAD50 is in the N-terminal half of the protein *(177).* IR-induced nuclear foci of BRCA1 substantially overlap induced foci of RAD50-MRE11-NBS1 *(159,177).* Curiously, damage-induced foci of BRCA1 seem to be of two distinct types; those that co-localize with RAD50-MRE11-NBS1, and those that colocalize with RAD51. Individual cells bearing both types of foci have rarely been seen *(106,177).*

Whether induction of nuclear RAD50-MRE11-NBS1 foci is dependent on BRCA1 is controversial. One group *(177)* found that in HCC1937 cells, damage-induced foci of BRCA1 were reduced and RAD50-MRE11-NBS1 foci were nearly undetectable. RAD50, MRE11, and NBS1 are expressed at approximately normal levels in HCC1937 cells, and could be co-immunoprecipitated with antibodies to RAD50, but these

immunoprecipitates did not capture the truncated BRCA1 protein. Transient transfection of HCC1937 cells with a vector expressing full-length BRCA1 restored radiation-induced formation of RAD50-MRE11-NBS1 foci. However, a second group *(159)* reported that damage-induced formation of RAD50-MRE11-NBS1 foci is approximately normal in HCC1937 cells, and is not augmented by expression of full-length BRCA1. BRCA2 probably is not required for the formation of damage-induced RAD50-MRE11-NBS1 foci; they form normally in Capan-1 cells *(173).*

4.4. Interactions of BRCA1 and BRCA2 with Other DNA Repair Proteins

In addition to RAD51 and RAD50-MRE11-NBS1, BRCA1 and BRCA2 associate with several other proteins involved in sensing or repair of DNA damage (Fig. 1 and Table 2). Both BRCA1 and BRCA2 can be co-immunoprecipitated with p53, and both influence transcription in vivo from genes bearing the p53 response element *(28,89,107).* BRCA1 stimulates transcriptional activation by p53 *(28,107),* while BRCA2 inhibits it *(89).* RAD51 enhances the inhibition of p53 transactivation by BRCA2. Interaction of BRCA1 with RAD50-MRE11-NBS1 has recently been proposed to occur within a supercomplex of protein complexes that function in DNA repair and replication, termed BASC (BRCA1-Associated genome Surveillance Complex) *(159).* BASC was defined by immunoprecipitation studies in which the majority of cellular BRCA1 was recovered in a large protein complex with mass exceeding 2 MD. The anti-BRCA1 immunoprecipitates may contain as many as 40 specifically associated proteins. Among the identified components are RAD50-MRE11-NBS1; ATM; the RecQ-type helicase BLM; three subunits of replication factor C (RPC); and the mismatch repair proteins MSH2, MSH6, and MLH1 (RAD51 and BRCA2 are apparently not present). In reciprocal immunopreciptation experiments, many of these proteins also interact with each other. Mutations in the *BLM* gene cause Bloom's Syndrome, which is characterized by chromosomal instability and predisposition to cancer. Co-immunoprecipitation of BLM with BRCA1 is especially robust, and BLM was seen by immunofluorescence to form nuclear foci, co-localizing with BRCA1, RAD50, and MRE11 nuclear foci, in cells treated with hydroxyurea to inhibit replication. The BLM-BRCA1-RAD50-MRE11 foci also colocalize with foci of PCNA, reinforcing the idea that BASC is a damage surveillance and repair supercomplex operating in conjunction with replication. Whether BASC exists in vivo as a stable, integrated surveillance and repair "machine," or rather as a dynamic system of transient interactions remains to be established.

5. BRCA1 AND BRCA2 IN TRANSCRIPTION-COUPLED REPAIR

BRCA1 copurifies with RNA polymerase II and other components of the basal transcription complex *(122),* an association that may be mediated by direct interaction between BRCA1 and RNA helicase A *(4).* These findings suggest a general function in transcriptional regulation, possibly related to transcriptional transactivation by the BRCT domain of BRCA1 *(29,101).* The evidence relating BRCA1 to functions in transcriptional regulation has been recently reviewed *(37).* However, although there is no evidence for a general transcription defect in BRCA1 mutants, recent evidence indicates that BRCA1 has a role in transcription-coupled repair (TCR) of DNA damage, the process whereby damage is preferentially repaired on the transcribed strand of actively expressed genes. TCR has been reviewed by Leadon *(78).*

Gowen and coworkers compared TCR of damage occurring within the dihydrofolate reductase (DHFR) gene in mouse ES cells bearing either wild-type BRCA1 or a targeted mutation that deletes amino acids 223-763 of BRCA1 *(53,54).* In $BRCA1^{+/+}$ or $BRCA1^{+/-}$ ES cells, TCR of oxidative damage inflicted by exposure to IR or hydrogen peroxide is observed much as it has been previously in other cell types. However, TCR of oxidative damage is absent in ES cells homozygous for the $BRCA1^{\Delta 223-763}$ allele. The role of BRCA1 in repair of oxidative damage is specific to TCR, because the overall efficiency of oxidative damage repair for the genome as a whole is not reduced in $BRCA1^{-/-}$ cells. Curiously, BRCA1 is not required for TCR of UV-induced damage. The TCR defect in $BRCA1^{\Delta 223-763}$ ES cells correlates with sensitivity to DNA-damaging agents, as measured in colony-formation assays. $BRCA1^{\Delta 223-763}$ ES cells are significantly more sensitive to IR and to hydrogen peroxide than wild-type or heterozygotes, but have no increased sensitivity to UV.

TCR has recently been examined in the BRCA1-mutant human cell line HCC1937. Abbott et al. *(2)* found that HCC1937 is extremely sensitive to IR, and that this hypersensitivity can be substantially relieved by transient or stable expression of a modified form of BRCA1 in which amino acids 702–834 have been deleted. Use of this modified BRCA1 was necessary because expression of full-length BRCA1 inhibits the growth of human mammary cells in culture *(62,133,145).* Like $BRCA1^{\Delta 223-763}$ mouse cells, HCC1937 cells lack TCR of radiation-induced oxidative damage. TCR was restored, in parallel with radiation resistance, by expression of the modified BRCA1 (Δ702–834) protein. In the same study, HCC1937 cells were examined for overall efficiency of DSB repair, by pulsed-field electrophoresis of whole cellular DNA after γ-irradiation. DSB repair was not obviously defective in HCC1937 cells, and showed only a very slight improvement in HCC1937 cells transfected with $BRCA1^{\Delta 702-834}$. The results imply that the IR sensitivity of HCC1937 is owing more to defective TCR than inability to repair DSBs.

It is not yet clear whether the requirement for BRCA1 in TCR of oxidative damage is direct or indirect. It may reflect a direct participation in damage sensing or repair, or alternatively, an indirect function as a transcriptional regulator of proteins required for these processes. Leadon *(78)* argued that a requirement for BRCA1 as a transcriptional regulator is less likely, because the genes activated would have to be specific for repair of oxidative damage, because BRCA1 is not required for TCR of UV-induced damage. Another possibility is that BRCA1 is required to recognize oxidative DNA damage on the transcribed strand of actively expressed genes and recruit repair proteins to the site. Long before the discovery of BRCA1, it was proposed that RNA polymerase stalled at a site of damage may be the signal that directs repair preferentially to the transcribed strand *(96).* The observed associations of BRCA1 with RNA pol II and RNA helicase A *(4,122)* are consistent with such a role.

BRCA2 also has a role in TCR, though perhaps a more limited one than BRCA1. In ES and MEF cells bearing an exon 27 truncation of BRCA2, TCR of damage induced by IR is reduced but not abolished, though TCR of thymine gylcols produced by hydrogen peroxide, as well as UV damage, appears unaffected (S. A. Leadon, personal communication).

6. BRCA1 AND BRCA2 IN HOMOLOGOUS RECOMBINATIONAL REPAIR

Reports that BRCA1 and BRCA2 physically associate and co-localize with RAD51 suggested that both proteins are involved in homologous recombination. Moreover,

BRCA1- and BRCA2-mutant cells exhibit phenotypic traits similar to two other mammalian cell lines known to be deficient for HRR; those mutated for XRCC2 and XRCC3. These traits include reduced cellular viability, a specific spectrum of sensitivities to DNA damaging agents, and gross chromosomal instability (*see* Subheadings 3. and 8.). The similarity of phenotypes between *BRCA1* and *BRCA2* mutations and mutations in the *RAD51*-related genes *XRCC2* and *XRCC3* strongly support the idea that BRCA1 and BRCA2 have critical involvements in HRR.

For BRCA1, there is now direct evidence for such a role. Moynahan et al. *(105)* used the same *BRCA1*$^{-223-763}$ ES cell line previously used to demonstrate a defect in TCR *(53)* to examine the effects of BRCA1 deficiency on two classes of homologous recombinational events: gene targeting and HRR of a chromosomal DSB. Relative frequencies of gene targeting at two different loci in *BRCA1*$^{-223-763}$ cells were reduced by 20 to 50-fold as compared to heterozygous controls, reflecting both decreased targeting and, in one case, increased nonhomologous vector integration. To assess HRR of a chromosomal DSB, a *neo* direct-repeat recombination substrate was installed in *BRCA1*$^{-223-763}$ cells and heterozygous controls by gene targeting. A chromosomal DSB was induced in vivo by expression of the highly site-specific endonuclease I-*Sce*I. DSB-induced HRR was reduced by five- to six-fold in the *BRCA1*$^{-223-763}$ cells as compared to heterozygous controls. The molecular analysis used in this study also allowed for a measurement of DSB repair by imprecise NHEJ, which was unaffected or even slightly increased by *BRCA1* mutation.

The defect in HRR in *BRCA1*-mutant ES cells can be placed in perspective by comparison to the defects caused by mutations in the *XRCC2* and *XRCC3* genes. *XRCC2* and *XRCC3* are members of the *RAD51* gene family *(84).* Hamster cell lines mutated for each gene have been examined using chromosomally integrated recombination substrates similar to those employed by Moynahan et al. In XRCC2-mutant cells, HRR of I-*Sce*I-induced breaks occurred at a low basal level, but increased some 100-fold upon complementation by expression of the human *XRCC2* gene *(68).* Similarly in XRCC3-mutant cells, the frequency of DSB-induced HRR increased upon complementation with the human *XRCC3* gene by 25 to 30-fold in one study *(112),* and by 34-fold to 200-fold in a second study *(25).* The apparently greater severity of defects in the *XRCC2*- and *XRCC3*-mutant hamster cells as compared to *BRCA1*$^{-/-}$ mouse cells should be interpreted with caution, as it could be due partly to the species difference, or to differences in genetic background (e.g. p53 status) and experimental design. Even so, it appears that the deficiency in HRR inflicted by loss of *BRCA1* function in mouse cells is comparatively moderate.

For BRCA2 as well, there is now direct evidence for a function in HRR. Two groups have independently reported that expression of BRCA2 fragments containing a single BRC repeat (the RAD51-interacting domains of BRCA2) results in hypersensitivity to DNA damage by IR, MMS or cisplatin, reduces formation of subnuclear RAD51 foci after IR exposure, and suppresses induction of the G2/M cell cycle checkpoint *(30,173).* These effects are presumed to come about by a dominant-negative mechanism, i.e. through competitive interference with the normal protein-protein interaction between BRCA2 and RAD51. The dominant-negative effect of BRC repeats has been exploited to assess the dependence of HRR upon the BRCA2-RAD51 interaction. A recombination substrate comprising inverted repeats of the *pac* (puromycin resistance) gene was

installed in HT1080 human fibrosarcoma cells, such that high frequencies of HRR occur following induction of a chromosomal DSB within one *pac* repeat using *I-SceI* endonuclease. When expression of I-*Sce*I in these cells is accompanied by expression of a BRC repeat peptide, HRR of the induced DSB is reduced by at least fifteen-fold compared to I-*Sce*I-only controls (Brenneman, Chen, and Nickoloff, unpublished results). Other types of homologous interactions are also affected by BRCA2 deficiency. In Capan-1 cells, the frequency of homologous recombination between extrachromosomal plasmid substrates increases by about eight-fold upon complementation with wild-type BRCA2, and gene targeting frequency is increased by more than ten-fold (S. N. Powell, personal communication).

7. BRCA1 AND BRCA2 IN CELL-CYCLE CHECKPOINTS

Normal cells exposed to acute DNA-damaging treatments such as IR respond by activating cell-cycle checkpoints. Activation of the G1/S checkpoint causes arrest at the G1/S transition, such that cells bearing unrepaired damage are prevented from beginning DNA synthesis until repair can be effected, while the G2/M checkpoint arrests cells that have sustained chromosomal damage during or after replication, preventing them from progressing to mitosis. It has been proposed that both BRCA1 and BRCA2 function in DNA damage-induced cell-cycle arrest, and that failure to restrain cell-cycle progression in the face of DNA damage may underlie the heightened sensitivity of BRCA1- and BRCA2-mutant cells to DNA damage, as well as their tendencies to genomic instability and neoplastic transformation. This in no way contradicts the idea that BRCA1 and BRCA2 function directly in DNA repair. There is growing evidence that both kinds of defects, repair and checkpoint, contribute to the cellular phenotypes and carcinogenic potential of BRCA1 and BRCA2 mutations. This is particularly so for BRCA1, which is better studied in this regard.

BRCA1 is evidently required for the G2/M damage-induced checkpoint *(46,77,166).* MEF bearing a targeted deletion of exon 11 in BRCA1 showed little reduction (or even an increase) in mitotic index after γ-irradiation, under conditions that reduced the mitotic index of wild-type cells by as much as 70% *(166).* The same study demonstrated that the checkpoint function of BRCA1 is specific to certain kinds of damage. The G2/M checkpoint response of BRCA1-mutant cells after exposure to UV light or MMS was not affected. This correlates in an interesting way with BRCA1 involvement in DNA repair, because BRCA1-mutant cells are defective for TCR of oxidative damage (a major product of γ-radiation) but not UV-induced photodamage *(53),* and impaired for HRR of DSBs (also a product of γ-radiation) *(105).* It appears that BRCA1 triggers G2 arrest only in response to types of DNA damage that it participates in repairing.

BRCA2 apparently is also required for the G2/M checkpoint. In a human tumor cell line that expresses normal BRCA2, overexpression of a BRCA2 fragment containing the fourth BRC repeat motif exerts a dominant-negative effect, as evidenced by increased sensitivity to γ-radiation and by a reduction in formation of radiation-induced RAD51 nuclear foci *(30).* Cells expressing the dominant-negative peptide exhibit little or no reduction in mitotic index after γ exposure, indicating abrogation of the G2/M checkpoint. Loss of G2/M checkpoint function was not seen in primary mouse cells with sublethal targeted truncations of BRCA2 *(103,109).* However, a different method of assessing G2/M checkpoint function was used in these studies (flow-cytometric scor-

ing of cells having 4N DNA content), and it is important to note that these are mutations intended to preserve some BRCA2 function.

The G1/S checkpoint is evidently not affected by BRCA1 or BRCA2 deficiency. Mouse cells homozygous for a targeted mutation of BRCA1 show a similar G1/S checkpoint to wild-type controls *(166),* and human cells expressing a dominant-negative BRCA2 fragment also show a wild-type G1/S checkpoint *(30).* That the G1/S checkpoint is intact in BRCA1- and BRCA2-mutant cells reinforces the idea that mutation of these genes must be accompanied by mutations in other checkpoint genes such as p53 in order for tumorigenesis to proceed *(39,79,129).* Although p53 mutations are generally common in human tumors, they are significantly more common in $BRCA1^{-/-}$ and $BRCA2^{-/-}$ tumors *(55,119).*

The loss of G2/M checkpoint function in BRCA1- and BRCA2-deficient cells suggests that, even in the absence of exogenous DNA damage, chromosomes bearing unrepaired spontaneous damage incurred in the course of replication will enter mitosis with abnormally high frequency. The resulting errors of segregation may underlie the gross chromosomal instability seen in mouse cells with mutations in *BRCA1* or *BRCA2 (24,129,152,166).*

8. BRCA1 AND BRCA2 IN MAINTENANCE OF CHROMOSOMAL STABILITY

Gross chromosomal instability has emerged as another feature characteristic of cells mutated for BRCA1 or BRCA2, and indicative of defects in cellular response to DNA damage. Cells of mouse embryos with a targeted deletion of *BRCA1* exon 11 ($BRCA1^{11-/-}$) have abnormal numbers of chromosomes, either greater or less than the normal number of 40, in up to 30% of metaphases examined *(129).* Wild-type embryos had numerical changes in only about 3% of metaphases. When the $BRCA1^{11-/-}$ mutation was bred into a checkpoint-defective ($p53^{-/-}$) background, the chromosome number abnormalities increased to over 70% of metaphases. Structurally abnormal chromosomes including translocations and dicentrics were also seen in mutant embryos. In culture, $BRCA1^{11-/-}$ primary MEF rapidly accumulate chromosome abnormalities, both structural and numerical *(166).* These cells are also defective for the G2/M cell cycle checkpoint (*see* Subheading 7.). Most remarkably, they frequently contain multiple centrosomes that give rise to multipolar mitotic spindles and aberrant chromosome segregation at cell division *(166).* This indicates that BRCA1, in addition to DNA repair and checkpoint functions, has some role in regulating centrosome replication. This role might be accomplished indirectly through the transcriptional regulatory functions that have been attributed to BRCA1. However, in monkey and human cells BRCA1 is localized to the centrosomes during mitosis, and physically interacts with γ-tubulin *(63),* which suggests that its effects are exerted directly, via protein-protein interactions.

BRCA2 mutation is also associated with gross chromosomal instability in mouse cells. Primary MEF with a targeted truncation of BRCA2 within exon 11 (retaining only three of the eight BRC motifs) had structural chromosome abnormalities, most notably chromatid breaks and chromatid exchanges, in about 40% of metaphases examined at the first passage after dissociation from embryos, increasing to almost 70% by the third passage *(109).* Curiously, the structural chromosome abnormalities in these

cells (as well as their growth defect in culture) can be largely suppressed by expressing dominant-negative mutant forms of p53 or the spindle-assembly checkpoint kinase Bub1 *(79).* Primary MEF bearing a milder truncation (retaining all the BRC repeats, but deleting the extreme carboxy-terminal RAD51-binding domain encoded by exon 27) similarly displayed elevated frequencies of structural chromosome abnormalities that increased with time in culture, such that 50–60% of metaphases were abnormal by the third passage *(24).* Spontaneously immortalized MEF with the same exon 27 deletion also showed evidence of ongoing chromosomal instability *(24).* The modal chromosome number in these cells was 60, intermediate to the normal diploid number of 40 and the approximately tetraploid chromosome numbers usually seen in newly immortalized mouse cells *(148).* Such sub-tetraploid chromosome numbers may reflect tetraploidization followed by chromosome loss. These cells were also conspicuous for the presence of one to several abnormally shortened chromosomes in every metaphase examined. By fluorescence *in situ* hybridization analysis, the majority of the abnormally small chromosomes either lacked telomeres or lacked a centromere, indicating that they are chromosome fragments. Chromosome fragments lacking a centromere are subject to loss by segregation errors, whereas fragments that lack telomeres can be expected to undergo progressive shortening, and further cycles of fusion and breakage. Dicentric chromosomes were also frequently seen, and may reflect the latter process.

Most recently, mutation of BRCA2 in mouse cells has been associated with centrosome amplification and segregation errors, much as for BRCA1 *(152).* Primary MEF with an exon 11 truncation of BRCA2 were found to show spontaneous micronucleus formation (indicating the presence of broken and/or incorrectly segregated chromosomes) in about 30% of cells at the second passage after dissociation from embryos, increasing to more than 50% by passage 3. Aneuploid chromosome numbers were present in more than 80% of metaphases examined, even at passage 2. Immunofluorescent staining with antibodies to γ- and β-tubulins revealed extra centrosomes and abnormal spindles in 44% of cells at passage 2, rising to 65% at passage 3.

In human cells, it is not yet clear whether the effects of BRCA1 or BRCA2 loss on chromosome stability are as severe as in mouse cells. The human BRCA1-mutant tumor cell line HCC1937 and the human BRCA2-mutant tumor cell line Capan- 1 both have highly aneuploid and variable karyotypes *(75,149),* but it cannot be known whether this was a cause or a result of neoplastic transformation (or both). More generally, breast cancers from BRCA1 and BRCA2 germ-line mutation carriers show markedly higher frequencies of chromosome rearrangements than sporadic breast cancers *(146,147).*

9. CONCLUDING REMARKS

The roles of BRCA1 and BRCA2 have begun to emerge in outline, though it is by no means certain that the activities identified so far are all that will ultimately be found. It is now clear that both proteins are involved in at least three types of function essential to the preservation of genomic integrity: DNA repair, cell-cycle checkpoint activation, and control of centrosome replication. The actions of BRCA1 and BRCA2 in chromatin remodeling and transcriptional regulation might subserve any or all of the aforementioned functions. Far less clear is how BRCA1 and BRCA2 participate in these functions at the molecular level. It seems likely that much of what BRCA1

and BRCA2 do, they do together. The similarity of mutant phenotypes in mouse support this view, as do the similar patterns of subnuclear localization and re-localization in response to DNA damage. The physical interaction and co-localization of both proteins with RAD51 also suggest that they participate in a single pathway, at least in regard to HRR. The involvement of both BRCA1 and BRCA2 in TCR suggests that they may function together in this repair pathway as well. That mutation of either BRCA1 or BRCA2 compromises activation of the G2/M checkpoint, but not the G1/S checkpoint, implies that they act together in signaling the presence of DNA damage in replicating chromosomes. The redundant centrosomes and abnormal chromosome segregation seen with mutation of either gene suggest that BRCA1 and BRCA2 also work together in control of centrosome replication. However, numerous interactions of BRCA1 and BRCA2 with other proteins have been detected (Fig. 1 and Table 2), as discussed in previous sections, and additional interactions probably await discovery. Full understanding of BRCA1 and BRCA2 will require a focus on their roles as members of multiprotein complexes.

The involvement of BRCA1 in both TCR and HRR suggests that it may perform analogous functions in the two repair pathways; possibly the sensing of DNA damage. The association of BRCA1 with the RNA polymerase II holoenzyme *(122)* may reflect such a function in TCR, serving to target repair to the template strand. If stalling of RNA polymerase at a lesion in the template strand is the signal that triggers TCR *(96),* then it can be imagined that BRCA1 acts to sense and transduce this event, and that it might sense and transduce the analogous event (stalling of DNA polymerase at a lesion) in the context of HRR or lesion bypass associated with replication. Though no direct association of BRCA1 with DNA polymerase has been uncovered, the co-localization of BRCA1 with the essential replication protein PCNA after DNA damage and its physical association with replication factor C are consistent with such a role *(123,159),* as is the recently reported ability of BRCA1 to bind DNA ends and nicks *(169).* But if the function of BRCA1 is to sense DNA damage, then how is the presence of damage transduced? Is the next event recruitment of DNA repair proteins? Direct activation of a checkpoint signaling cascade? Indirect activation of repair or checkpoint functions via transcriptional regulation? BRCA1 may function in a combination of these processes.

The robust physical association of BRCA2 with RAD51 and the iterated RAD51-binding domains in BRCA2 imply an intimate functional interaction. Targeted truncations of BRCA2 in mouse suggest that all of the RAD51-binding domains of BRCA2 are required for a fully functional interaction. One potential explanation for this is that the relevant ligand of BRCA2 is not monomeric RAD51, but rather multiple units of RAD51 assembled with single-stranded DNA into a nucleoprotein filament. In this scenario, BRCA2 might function to facilitate the loading or compacting of RAD51 monomers onto single-stranded tails produced by processing of DSBs. Alternately or additionally, BRCA2 might signal the presence of RAD51-nucleoprotein filaments (and hence that HRR is active) through interaction with BRCA1, via the transcriptional activation domain in its amino-terminus, or via other functional domains yet to be identified in this enormous protein.

Many of the identified involvements of BRCA1 and BRCA2 relate directly to cell proliferation, and this is consistent with the growth defects associated with *BRCA1* and *BRCA2* mutations in mouse cells and embryos. Defects in homologous recombi-

nation occurring in conjunction with replication may be the proximate cause of growth impairment. *In E. coli,* recombinational processes are required for repair and bypass of template defects during replication and for the restart of collapsed replication forks (*42,71,72; see* also Chapter 2). A growing body of evidence supports the conservation of this requirement in eukaryotes *(108,144).* Frequent errors of chromosome segregation may also contribute to growth impairment, by rendering the products of many cell divisions inviable. However, the association of growth defects with BRCA1 and BRCA2 mutations poses a paradox in relation to carcinogenesis: how can mutations that severely impair cell proliferation promote development of a tumor? It has been proposed that loss of BRCA1 or BRCA2 can promote carcinogenesis only if it is accompanied by mutations that disable other checkpoint and apoptotic functions *(55, 61, 79).* This raises the question of whether loss of BRCA1 or BRCA2 acts as an initiating event in carcinogenesis, by causing genomic instability that drives the loss of other growth-controlling genes, or whether loss of BRCA1 or BRCA2 is more usually a secondary event, contributing to carcinogenesis in cells that have already lost the ability to arrest or self-destruct in the face of accumulating genomic damage.

Regardless of whether it is typically the first or a later event, it is probable that mutation of BRCA1 or BRCA2 promotes tumorigenesis by causing genomic instability at the chromosomal level, i.e., deletion and rearrangement of chromosome parts, and loss or gain of entire chromosomes. In mouse cells, the chromosome instability associated with *BRCA1-* and *BRCA2* mutation now appears to be driven by three defects operating in conjunction: failure to repair DNA damage that arises during or after replication; failure to arrest cell-cycle progression at G2 in the presence of unrepaired damage and; and failure to correctly segregate chromosomes during mitosis owing to unrepaired chromosome breaks and centrosome/spindle malfunction. To the extent this genome-destabilization scenario holds in human cells, it may go far in explaining the carcinogenic effect of *BRCA1* and *BRCA2* mutations. In this regard, both BRCA1 and BRCA2 fit well into the category of "caretaker" genes, as conceptualized by Kinzler and Vogelstein *(70).*

An important question still outstanding is why loss of BRCA1 or BRCA2 in humans is associated predominantly with breast and ovarian cancers. Their roles in DNA repair, checkpoint induction, and regulation of centrosome function would seem equally important in any rapidly proliferating cell population. Why then do tumors arise with highest frequency in estrogen-responsive tissues? One possibility is suggested by the observation that BRCA1 can restrain the growth of normal and malignant breast epithelial cells, but not tumor cells derived from other tissues *(145).* It may be that BRCA1 (perhaps in conjunction with BRCA2) has been recruited to perform a growth-regulating function specific to the developmental program of breast and ovary, i.e., modulation of the mitogenic stimulus of estrogen. BRCA1 can inhibit signaling by the ligand-activated estrogen receptor (ER-α) and block its transcriptional activation function *(45).* Another possibility, raised by Livingston and coworkers *(33,34),* is that certain metabolic products of estrogen, by forming DNA adducts, add to the load of endogenous DNA damage in cells that concentrate estrogens. Either or both of these possibilities, added to the general effects of BRCA1 or BRCA2 deficiency on DNA damage response and genomic integrity, might account for the heightened risk of cancer in breast and ovary.

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11

DNA Repair and the Generation of Immune Diversity

The Agony and the Ecstasy

Lauryl M. J. Nutter, Chrystal K. Palaty, Martin Nemec, Cynthia J. Guidos, and Jayne S. Danska

1. INTRODUCTION

Lymphocytes endow the immune system with the capacity to specifically recognize and destroy a vast array of pathogenic microorganisms. To accomplish this task, lymphocytes express clonally unique antigen receptors that transduce signals that activate humoral (B cell) or cellular (T cell) effector functions upon recognition of a suitable antigenic ligand. Lymphocytes require the faithful execution of DNA repair processes first to generate, and then to refine the recognition specificity of their antigen receptors. Antigen receptor diversity is generated somatically during lymphocyte development through a DNA breakage and rejoining process called V(D)J recombination. Site-specific recombination of variable (V), diversity (D), and joining (J) gene segments assembles the variable region exon of an antigen receptor chain in each newly generated lymphocyte. In mice and humans, substantial germline diversity exists in tandem genomic arrays of dozens to hundreds of each gene segment. The random recombination of particular V, D, and J segments in immature lymphocytes combinatorially expands this germline repertoire. Importantly, imprecision in the joining process further diversifies the available germline repertoire by several orders of magnitude. V(D)J recombination occurs early in lymphocyte development, but more mature B cells undergo V gene hypermutation and/or additional rounds of V(D)J recombination to further refine their antigen specifities. Finally, the immune effector function of the immunoglobulin (Ig) heavy chain can be modified by class-switch recombination, a transaction involving targeted, but not site-specific, breakage and rejoining of different constant region genes. Thus, lymphocytes are clearly unique in their dependence on multiple DNA repair processes for their maturation and function. Defects in these pathways can result in profound immune deficiencies. Furthermore, genetic rearrangement and hypermutation occur in lymphocytes that have great proliferative potential, so infidelity in these processes poses a risk of genomic instability and growth dysregulating

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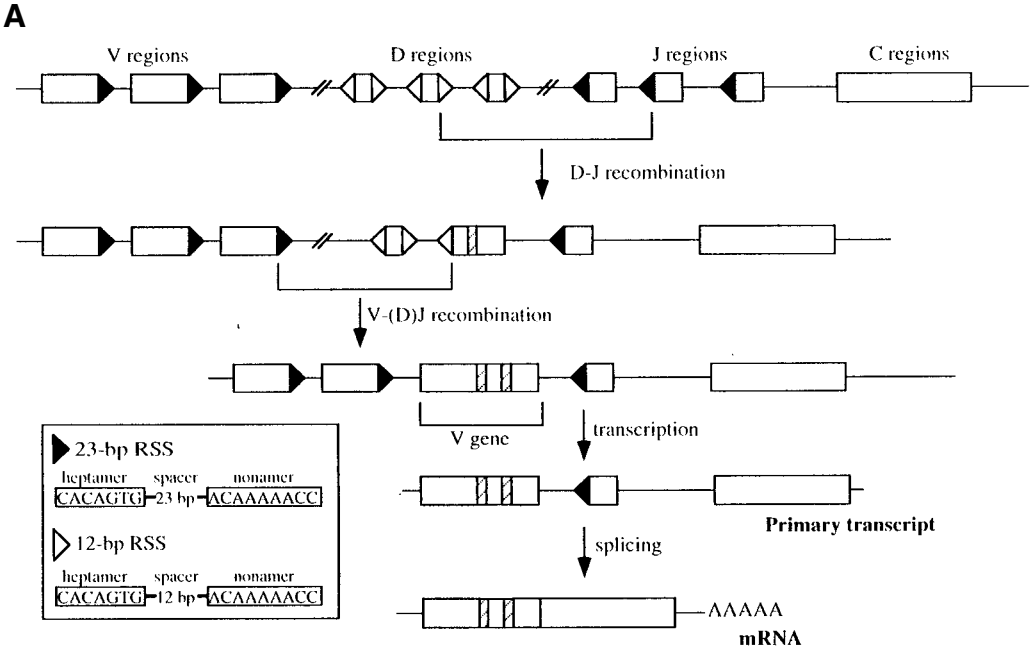
mutations. DNA damage checkpoints help to ensure that DNA breakage and rejoining at antigen-receptor genes are carefully controlled, and to eliminate lymphocytes containing potentially dangerous mutations. This chapter will explore the molecular regulation of V(D)J recombination and other physiological DNA repair mechanisms operative in lymphocytes, emphasizing recent, and sometimes conflicting, data in a rapidly expanding field of DNA repair. The pathological consequence of defects in these processes will also be addressed in the context of rodent models and human disease. We hope that the reader will come to appreciate the complex molecular choreography required to create, modify, and deploy antigen-reactive lymphocytes, processes that are not yet fully understood.

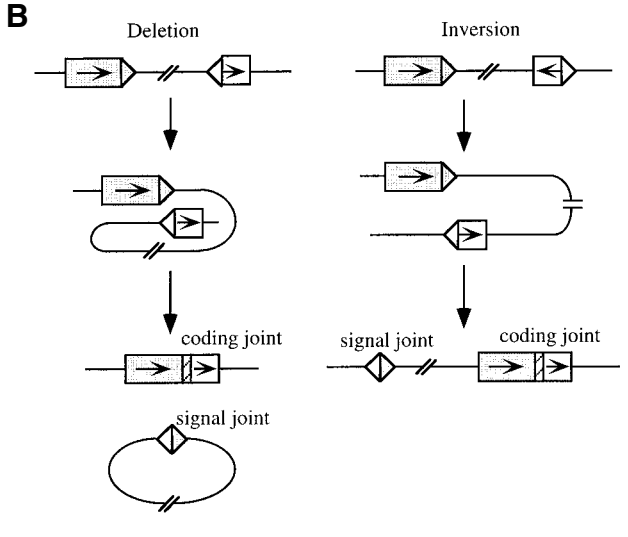
2. LYMPHOCYTE DEVELOPMENT

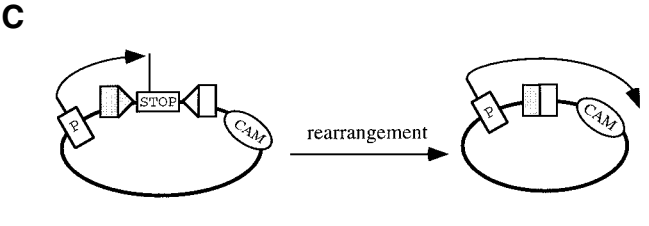
2.1. Structure of Antigen-Receptor Proteins and Genes

Most T cells express on their surface a T cell antigen receptor (TCR) consisting of disulfide-linked α and β chains, but a minor T-cell sublineage expresses an alternative TCR, composed of a γδ heterodimer. TCR-αβ and some TCR-γδ recognize cell-bound antigen fragments complexed with major histocompatibility complex (MHC) proteins. In contrast, the B-cell antigen receptor (BCR) is a membrane-bound Ig that is able to capture soluble antigens. Monomeric Ig is a dimer of dimers, consisting of two Ig heavy (IgH) chains and two Ig light (IgL) chains. Each IgL chain is disulfide linked to one IgH chain, and the two IgH chains are disulfide-linked to each other. There are two IgL isotypes (κ, λ) generated from distinct genetic loci, but each B cell only expresses one IgL isotype. Following ligand binding by the IgH/IgL and TCR-αβ or TCR-γδ heterodimers, a noncovalently associated invariant polypeptide complex (Ig-αβ and CD3, respectively) transduces intracellular signals to activate immune effector functions *(168,198).* The clonally unique N-terminal variable region of both antigen-receptor chains determines antigen specificity in T and B cells. The constant regions mediate effector functions and/or structural roles. Most antigen receptor loci encode only one or two functionally identical constant (C) region genes. However, there are many IgH C region genes (μ, δ, γ1–4, α, ε), and class-switch recombination between them generates multiple IgH subclasses that mediate diverse effector functions (Subheading 7.3).

Fig. 1. Schematic representation of V(D)J recombination. **(A)** V(D)J recombination involves the site-specific recognition and cleavage of chromosomal DNA by lymphoid-specific proteins (RAG1/2), to bring variable (V), diversity (D), and joining (J) gene segments of antigen receptors together to create functional transcripts. The site-specific cleavage occurs at recombination signal sequences (RSSs) represented here by shaded triangles. The inset panel shows the DNA sequence of the RSSs. **(B)** Two types of V(D)J recombination can occur: deletional and inversional. Deletional rearrangement occurs between gene segments in the same transcriptional orientation. Inversional recombination occurs between gene segments in opposite transcriptional orientation. Transcriptional orientation is indicated by the arrows within gene segments. **(C)** Extrachromosomal recombination substrates (ECRSs) are used to assay V(D)J recombination in cell culture. Prior to recombination, the two RSSs flank a transcriptional terminator (STOP). Transcription is initiated at a bacterial promoter (p), but is interrupted by the terminator. Successful RSS-mediated rearrangement deletes the terminator and transcription of a marker gene, most often encoding resistance to chloramphenicol (CAM), occurs allowing selection of the rearranged product.


A
V regions
D regions
J regions
C regions
D-J recombination
V-(D)J recombination
V gene
transcription
Primary transcript
splicing
AAAAA
mRNA
23-bp RSS
heptamer
spacer
nonamer
CACAGTG
23 bp
ACAAAAACC
12-bp RSS
heptamer
spacer
nonamer
CACAGTG
12 bp
ACAAAAACC


B
Deletion
Inversion
coding joint
signal joint
signal joint
coding joint


C
P
STOP
CAM
rearrangement
P
CAM

The organization of the antigen receptor genes reveals how a limited set of genes codes for receptors capable of binding an enormous variety of antigens (Fig. 1A). Variable region genes are encoded by clusters of discontinuous gene segments, spread over vast regions of a chromosome (reviewed in *149).* Somatic rearrangement brings these gene segments together in the process of V(D)J recombination. TCR-β, TCR-δ, and IgH V regions consist of V, D, and J gene segments, while only V and J gene segments comprise TCR-α, TCR-γ, and IgL V regions. Thus, each antigen receptor contains a V(D)J chain bound to a VJ chain. The developmental sequence in which the gene segments are rearranged is highly ordered. D and J segments (of TCR-β and IgH loci) are generally joined first, and then a V segment is joined to the DJ segments, yielding a template for RNA transcription. As the gene segments are connected, nucleotides may be removed or added in a non-templated manner at the joint. The processes of somatic DNA rearrangement and imprecise DNA joining create a diverse repertoire of antigen receptors, enabling the detection and clearance of a myriad of infectious agents.

It should be pointed out that gene rearrangement is not the major mechanism for diversifying the germline repertoire in all animals (reviewed in *167,293*). Chickens have a single functional IgH and IgL V element, resulting in a rather limited primary B-cell repertoire. Interestingly, this repertoire becomes highly diversified following antigenic stimulation of mature B cells by a gene-conversion process. The donor sequences are provided by a large family of highly homologous and clustered pseudo-V_H and pseudo-V_L genes located upstream of the functionally rearranged V_H or V_L gene. Rabbits have approx 200 V_H gene segments, but only about half are functional, and the initial rearrangement in most B cells is subsequently diversified using functional and nonfunctional upstream V_H elements. Superficially, V region diversification by gene conversion seems similar to the mating-type switch in *Saccharomyces cerevisiae.* Though it seems likely that targeted gene conversion of V elements involves the generation of double-strand DNA breaks (DSBs), a site-specific endonuclease, analogous to the HO endonuclease that initiates the mating-type switch in yeast, has not been identified.

2.2. *Regulation of Lymphocyte Development by V(D)J Recombination*

The tremendous somatic diversification of the germline repertoire that is afforded by V(D)J recombination comes at a high price. Because of the variable nucleotide loss or addition at V(D)J junctions, each joining event has only a one-third chance of being in-frame. Because generating both chains of an antigen receptor requires at least 5 joining events, most developing lymphocytes will fail to express a functional antigen receptor, even if they attempt rearrangement on both chromosomes. The evolutionary solution was to connect intimately lymphocyte development to the timing and success of V(D)J recombination. Accordingly, antigen receptor-mediated developmental checkpoints regulate the development of T and B lymphocytes in similar ways (reviewed in *119,287).* Recombination is first activated at the IgH and TCR-β loci in pro-B or pro-T cells, respectively (Fig. 2). Successful (in-frame) rearrangement allows expression of pre-BCR or pre-TCR in which the IgH/TCR-β protein is paired with a "surrogate" IgL or pre-TCR-α chain encoded by nonrearranging genes. Expression of these surrogate chains is limited to immature lymphocytes, and contributes to the assembly of pre-BCR or pre-TCR complexes, which signal progenitors to cease rearrangement, proliferate extensively, and to develop to the pre-B or pre-T stage where rearrangements of IgL and

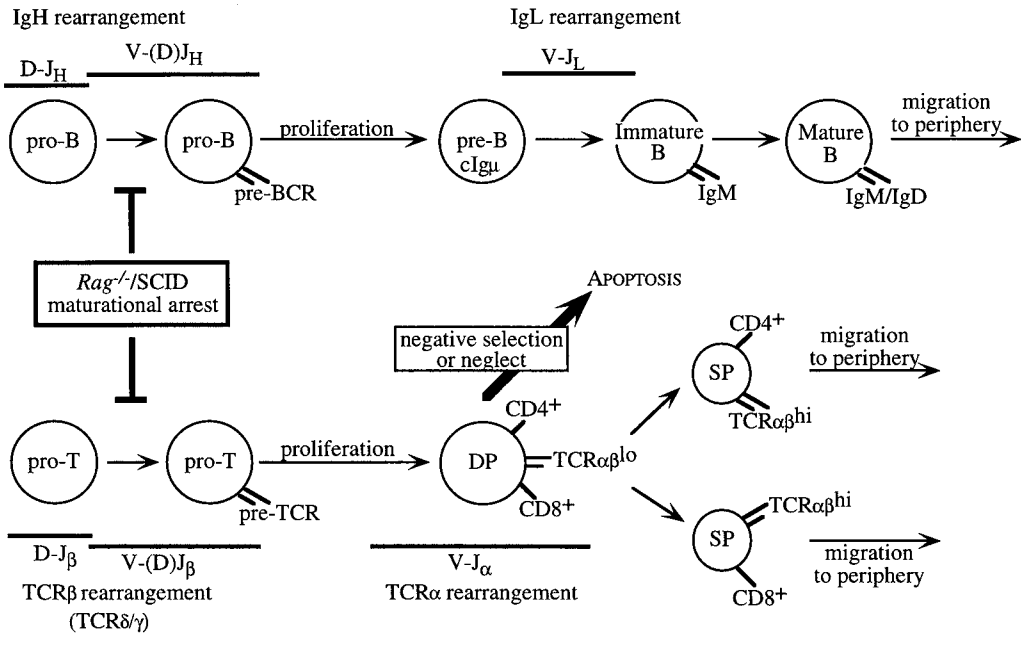


Fig. 2. Schematic representation of B- and T-lymphocyte development. Productive rearrangement of IgH or TCR-β genes and signaling through the pre-BCR and pre-TCR, respectively, stimulates precursor proliferation, allelic exclusion of unrearranged IgH or TCR-β, initiation of IgL or TCR-α rearrangement, and subsequent maturation. RAG-deficiency or the SCID mutation arrests development at the pro-B or pro-T cell stages, though the mechanistic reason for arrest is different. *See* Subheading 2.2. for details.

TCR-α loci begin. Thus, this first developmental checkpoint serves to clonally expand the rare progenitors that successfully rearrange IgH or TCR-β, and to induce their developmental progression to the next phase of rearrangement. Because of the random nature of V(D)J recombination, most pre-T cells will express TCR-αβ that do not recognize foreign antigens complexed with self-MHC proteins, and are useless to the immune system. A second developmental checkpoint, known as positive selection, ensures that only pre-T cells expressing self-MHC-specific TCR-αβ mature into helper and cytotoxic T cells *(87).* B cells expressing IgH/IgL with high affinity for foreign antigens are also positively selected, but only after they enter the peripheral circulation (Subheading 7).

The vast majority of developing lymphocytes (90–99%) is eliminated by apoptosis before completing their development (Fig. 2). Although it is clear that some of this loss is accounted for by precursors failing to make functional TCR or Ig rearrangements, a second developmental consequence of V(D)J recombination is that it has the capacity to generate antigen receptors specific for self, rather than foreign, antigens. To minimize the potential for autoimmunity, a receptor-mediated negative-selection process induces apoptosis in immature (or sometimes mature) T and B cells with high-affinity autoreactive BCR or TCR *(97,138).* Thus, diversification of the repertoire by V(D)J recombination has necessitated that developing lymphocytes evolve strategies for coping with the inefficiency of generating functional receptors, and the danger of generating autoreactive ones.

Dramatic evidence for the pivotal role of V(D)J recombination in lymphocyte development was provided by studies of mice homozygous for the spontaneous severe combined immune deficiency (SCID) mutation and mice bearing targeted deletions of either of the recombination activating genes, *Rag1* or *Rag2* (Subheading 3.). B and T lymphocyte development in SCID, RAG1-and RAG2-deficient mice is arrested owing to failure to generate pre-BCR or pre-TCR *(47).* Because developmental arrest occurs prior to the proliferative expansion elicited by pre-BCR and pre-TCR signals, RAG-deficient and SCID mice contain drastically reduced numbers of immature and mature lymphocytes, rendering the animals susceptible to opportunistic infections. Although the cellular basis of immune deficiency is largely indistinguishable in RAG-deficient and SCID mutant mice, the mechanistic failure in V(D)J recombination is distinct in these two models, and provides insight into the molecular steps in this DNA breakage and repair process, as discussed below.

3. BREAKING IT UP: THE V(D)J CLEAVAGE REACTION

V(D)J recombination is a site-specific, but nonconservative, DNA cleavage and repair process that apposes antigen-receptor gene segments (Fig. 1A). Lymphoid-specific endonucleases, RAG1 and RAG2 initiate this process (Fig. 3). The RAG proteins recognize recombination signal sequences (RSSs) flanking the V, D, and J gene segments, and a synaptic complex is formed between two segments. DSBs are introduced at the boundary between the RSS and the V, D, or J coding segment, generating two types of intermediates: hairpin-terminated coding ends (CEs) and blunt recombination signal ends (SEs). As discussed in Subheading 4, a complex of DNA repair proteins mediates CE ligation to form coding joints and SE ligation to form signal joints. V(D)J recombination can occur either by deletion or inversion, allowing recombination of V gene segments in either transcriptional orientation relative to the (D)J germline elements (Fig. 1B). Deletional rearrangement occurs between two gene segments in the same transcriptional orientation flanked by RSSs in opposite (head to head) orientations. This process generates a coding joint between germline elements, and excision of the intervening region, which is then religated to form an extrachromosomal circle (reviewed in *149).* Inversional recombination occurs between two gene segments in opposite transcriptional orientations that are flanked by RSSs in the same (head to tail) orientations. In this case, both coding and signal-joint formation occur on chromosomal DNA, and both are required to re-establish a patent chromosome.

V(D)J recombination can result in two types of "nonstandard" V(D)J products, called hybrid and "open-and-shut" joints. Open-and-shut joints result from the cleavage and reunion of adjacent CE and SE, whereas hybrid joints result from the cleavage and joining of a CE from one gene segment with the SE from a different segment (reviewed in *76,149).* Although rarely observed, these nonstandard events can apparently rejoin DSBs in a RAG-dependent fashion, without a full complement of repair proteins *(183).*

3.1. RSSs Mark the Spot: Recombination Signal Sequences

RSSs are evolutionarily highly conserved, and are absolutely required for V(D)J recombination. The RSS consists of highly conserved palindromic heptamers and conserved A/T-rich nonamers separated by a spacer region of either 12 or 23 base pairs (bp) in length (Fig. 1A, inset panel; *149).* Both in vitro and in vivo studies have demon-

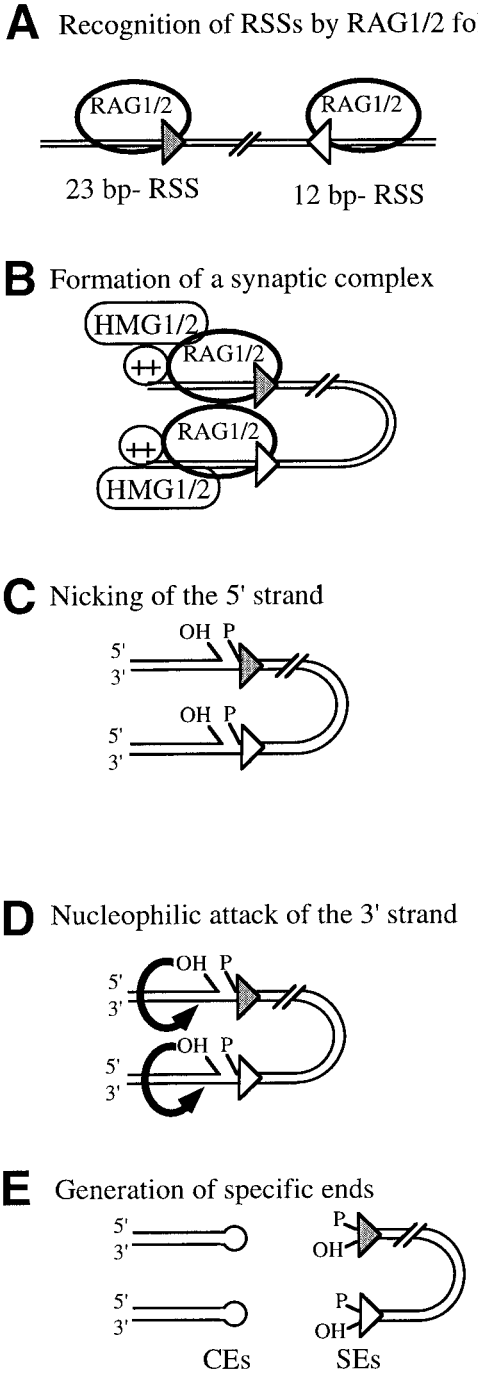


Fig. 3. The V(D)J recombination cleavage reaction. **(A)** RAG proteins (represented by ovals) recognize and bind to RSSs. The stoichiometry of RAG1/2 complex binding to the RSSs is not known. One tetramer may recognize both RSSs or different RAG1/2 tetramers may recognize different RSSs. **(B)** A synaptic complex forms, containing the RSS, RAG1/2 proteins, HMG proteins, and divalent cations. **(C)** The 5′ strand of the RSS is nicked by the RAG1/2 proteins. For simplicity, the synaptic proteins are not shown. **(D)** Nucleophilic attack by the hydroxyl group breaks the phosphodiester bond of the 3′ strand, creating a hairpin loop. **(E)** Covalently closed hairpin CEs and blunt 5′-phosphorylated SEs, the intermediates of V(D)J recombination, are the result.

strated that although the sequence of the spacer is not important, the length is crucial *(4,45,124,219,222,283,292).* The phasing of the spacer on the DNA helix may correctly position the nonamer and heptamer for binding by the RAG proteins. Recombination only occurs between a coding segment flanked by a 12-bp spacer RSS and a second one flanked by a 23-bp spacer RSS, a selection know as the "12/23 rule." Site-recognition, DSB formation, and rejoining of the gene segments are influenced by both RSS and the adjacent coding-segment sequences *(4,45,52,102,106,156,193,222,232,261,264,284).* Cellular assays and cell-free reactions using naked DNA templates containing RSSs with point mutations have highlighted critical RSS residues for each of these steps *(76,222,264).* Superimposed upon the influence of RSS and coding-sequence flanks, the timing and locus specificity of V(D)J recombination in developing lymphocytes is controlled by the chromatin conformation surrounding the antigen-receptor gene clusters.

3.2. Lymphoid Specificity and Accessibility

Extrachromosomal recombination substrate (ECRS) assays have been extremely important for dissecting many aspects of the V(D)J recombination process *(102,103,152,153,163).* These specialized shuttle vectors, containing both eukaryotic and prokaryotic origins of replication, are used to examine the frequency and fidelity of V(D)J recombination under readily manipulable conditions (Fig. 1C). The eukaryotic cells of interest are transfected with ECRS plasmids and, if necessary, with *Rag1/Rag2* expression constructs. Extrachromosomal DNA is harvested 48 hours later and transformed into *Escherichia coli.* The ECRS substrates contain 12 and 23 RSSs flanking a transcription terminator lying upstream of a prokaryotic promoter driving a chloramphenicol resistance (CAM) gene. Recombination at the RSSs deletes the terminator allowing expression the CAM gene, and survival of bacteria harboring recombined ECRS following CAM selection. Depending on RSS orientation, deletional and/or inversional recombination events can be assessed, and manipulation of the RSS and flanking coding sequences can reveal details of their influence on the process. These assays have been instrumental in revealing aspects of lineage and developmental specificity in the regulation of V(D)J recombination.

V(D)J recombination at each antigen-receptor locus displays lymphoid cell-type specificity, developmental-stage specificity, and a highly regimented order of rearrangement steps for each antigen-receptor locus *(149,246,262).* Lymphoid specificity of V(D)J recombination is achieved in part by restricted expression of RAG1 and RAG2 proteins in lymphoid precursors *(200,245).* Fibroblasts transfected with *Rag1* and *Rag2* expression constructs can recombine ECRS but fail to recombine their endogenous antigen-receptor loci, indicating additional levels of control on this process *(200,245).* Using intact nuclei from T- or B-lymphoid precursors of defined maturational stage, chromatin accessibility at each locus was shown to control the imposition of DSBs at RSSs *(262).* The cell-type and developmental stage-specificity of particular loci accessible to cleavage observed in the purified nuclei were identical to those observed in these cells in vivo *(262).* It should be noted that some cross-lineage V(D)J recombination can occur, because IgH rearrangements can be found in T cells, and TCR-β rearrangements are occasionally seen in B cells. However, these rearrangements are always limited to D-J joining and are usually out of frame. Thus, the lineage specificity of IgH vs TCR-β rearrangement is imposed at the V to DJ joining step in T- and B-cell precursors.

Changes in chromatin accessibility presumably reflect higher-order changes in chromatin structure that occur during lymphocyte development to regulate which RSSs are targeted by the recombinase proteins at each developmental stage *(248,256,262).* Locus accessibility to the recombinase is likely modulated by the binding of trans-acting factors to *cis*-acting regulatory sequences, such as transcriptional promoters *(253,299)* and enhancers *(99,180).* These elements may alter chromatin accessibility and influence recruitment of the RAG proteins *(180a,248,299)* and other repair components *(99).* Transcriptional activity at the locus may regulate accessibility, given observations that germline transcription often correlates with V(D)J recombination at a given locus *(98,256).* However, germline transcription is not absolutely required for V(D)J rearrangement of some V genes in the Igμ locus *(5).* Recently, the positioning of the RSS on the nucleosome and association of the high mobility group protein 1 (HMG1) have been shown to influence locus accessibility *(135).* Other DNA binding proteins, *cis*-acting elements, and additional molecular mechanisms may be involved in regulating accessibility *(98,248,256),* continuing to make this an intriguing area of study.

3.3 Structure of RAG proteins

Much is known about the function of the RAG proteins, owing to recent success at reconstituting the V(D)J recombination reaction in vitro using purified proteins. A major technical hurdle in establishing "V(D)J in vitro" was producing large amounts of purified RAG1 and RAG2 proteins, both of which are stubbornly insoluble when expressed as full-length bacterial fusion proteins. Deletion analysis identified core regions of RAG1 and RAG2 that retained RSS recognition and cleavage activity in vitro and in ECRS assays, and had greater solubility than the full-length proteins *(46,238,254).* The RAG1 core protein includes residues 384–1008 (N-terminal deletion of approx 30% of the protein) and the RAG2 core protein includes residues 1–387 (C-terminal deletion of approx 25% of the protein; *237,238).* However, the recombination efficiency of the full-length RAG proteins exceeds that of RAG core domains in ECRS assays *(177,265)* and on intact chromosomal substrates in vivo *(233).* Additional evidence that the RAG core regions are functionally essential has come from identification of mutations affecting these regions that cause severe combined immune deficiencies in humans *(249,286).*

Numerous motifs in the RAG proteins are thought to contribute to protein-protein or protein-DNA interactions. The most extensively studied is a helix-turn-helix DNA binding motif in the RAG1 core domain, which binds to the RSS nonamer in vitro *(52,261).* Other regions of RAG1 also influence DNA binding *(72,177,239).* RAG1 contains basic amino acid motifs that mediate binding to proteins involved in nuclear import *(42,44,147,260),* as well as other domains that may be required for interactions with other proteins *(229,233).* RAG2 also has potential protein-binding motifs *(237),* and several well-characterized phosphorylation sites, some of which affect cell-cycle regulation of RAG2 *(81,164,165,260).* Interestingly, the C-terminal region of RAG2 that is missing from the core protein has been implicated in the V_H to DJ_H joining step in vivo *(126).* It has been suggested that efficient disassembly of the post-cleavage complex and locus targeting require functions located outside of the RAG core domains *(195,265).* Further analysis of the full-length RAG proteins is needed to understand the protein partners and DNA interactions operating in V(D)J recombination.

3.4. RAG-Mediated DNA Cleavage

3.4.1. Recognition and Binding of the RSS

The first step of V(D)J recombination is the recognition of the RSS by the RAG proteins and formation of the synaptic complex (Fig. 3). In vitro studies have defined the initial recognition and binding of RAG1 within the minor groove of the RSS nonamer *(52,193,261,267)* and interaction with additional nucleotides in the spacer *(193).* These binding interactions occur through the RAG1 helix-turn-helix DNA binding motif *(52,261).* Interactions between RAG1 and the heptamer *(52,106,222,261,267)* and the coding flank appear to stabilize RSS-RAG interactions *(106).* Although RAG2 is absolutely required for cleavage at the RSS/coding border, direct binding of RAG2 to DNA has not been demonstrated *(3,52,193,261).* However, there is good evidence that RAG2 binding stabilizes RAG1/DNA interactions *(3,156,267).* Both RAG proteins can be isolated in a high molecular-weight complex, and the presence of RAG2 in this complex is dependent on the presence of RAG1 *(147).* The regions of RAG1 required to interact with RAG2 have been defined *(179),* and it has been demonstrated that they exist as a mixed tetramer with two of each molecule in solution and bound to DNA *(8a).* Although much remains to be discovered about these interactions, it is clear that both proteins are required for RSS binding and for formation of the synaptic complex.

3.4.2. Formation of the Synaptic Complex

It has long been postulated that preceding the recombination of two coding elements, their RSSs are physically apposed in a synaptic complex. This notion has been validated by recent studies in cell-free systems (Fig. 3B). This DNA-protein complex includes 12-bp and 23-bp spacer RSSs, the RAG1 and RAG2 proteins, a divalent cation, and likely HMG1 and HMG2, which may structurally distort or bend the DNA at the 23-bp spacer *(57,76,82,105,106,124,156,176,180,244,281,283,295).* The structural distortion introduced by HMG1/2 may potentiate RAG binding and recognition of the 23-bp spacer, permit the RAG proteins to distinguish 12-bp from 23-bp spacers, promote interactions with other proteins *(244),* and/or partially unwind the DNA revealing single-stranded DNA regions *(124).* Participation of numerous additional proteins in the process is likely *(244).* Assembly of this synaptic complex occurs before *(124,156,267)* and persists throughout cleavage, perhaps to tether the DNA ends *(2,57,82,105).*

3.4.3. DNA Cleavage Reaction

After assembly of the synaptic complex, RAG1 and RAG2 cleave at the RSS heptamer/coding sequence border in two successive steps. Cleavage yields four DNA ends: two hairpin-terminated CEs and two blunt SEs (Fig. 3C–E; *176).* Extensive in vitro analyses have defined essential roles for both RAG1 and RAG2 during cleavage *(3,57,106,156,176,200,282).* First, the RAG proteins act as an endonuclease to nick one strand of DNA at the boundary between the coding sequence and the 5′ end of the RSS heptamer, yielding a 3′-hydroxyl group. In the second step, this 3′-hydroxyl group attacks the phosphodiester bond of the complementary DNA strand through trans-esterification. Under suitable in vitro conditions, cleavage at the 12-bp and 23-bp RSSs occurs almost simultaneously in a coupled reaction *(57,284),* generating a covalently closed hairpin CE and a blunt 5′-phosphorylated SE *(176,220,283).* Though recent data

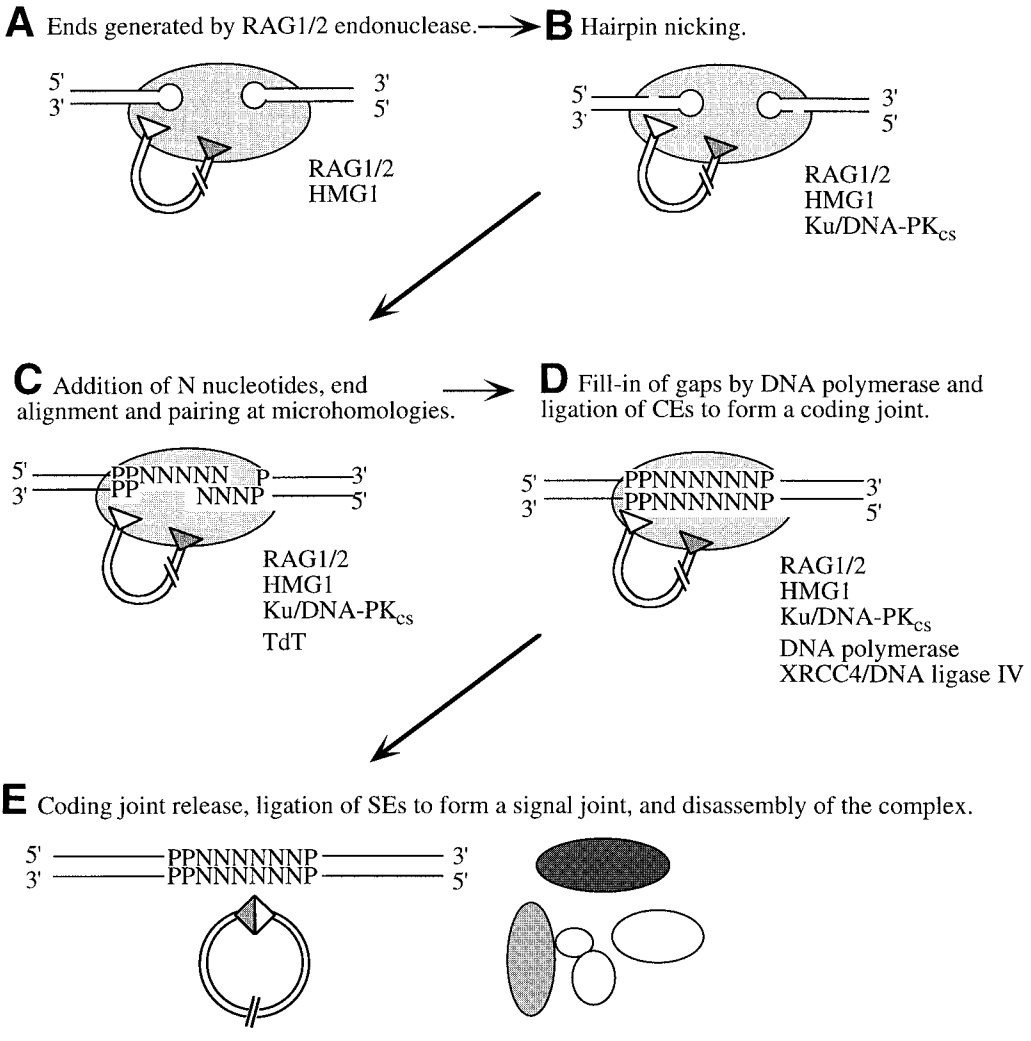


Fig. 4. A model of end-joining in V(D)J recombination. Ligation of V(D)J ends into coding and signal joints is a carefully regulated multistep process involving many proteins. The gray oval represents a multiprotein complex involved in V(D)J joining. **(A)** Ends generated by the RAG1/2 endonuclease are held together in a postcleavage complex. **(B)** The recruitment of "joining factors" facilitates hairpin nicking and opening. This process may be carried out by RAG itself. **(C)** Once opened, CEs are processed with the addition of N nucleotides by TdT, followed by end-alignment, and pairing at microhomologies. **(D)** Prior to ligation by DNA ligase IV, gaps must by filled in by a DNA polymerase. **(E)** Release of the coding joint may precede signal-joint formation, which is quickly followed by the disassembly of the complex. Spatial relationships are neither known nor implied by this figure. *See* Subheading 6. for further details.

implicate the formation of a synaptic complex in vivo, under physiological conditions low levels of single RSS cleavage can be seen at nonconsensus RSSs, suggesting that the 12/23 rule can sometimes be violated in vivo *(264).* This observation, coupled with the suggestion that the genome may be littered with nonconsensus, or "cryptic," RSSs *(151),* implies that proper regulation of the cleavage reaction may be crucial for limiting oncogenic translocations in lymphoid precursors.

3.4.4. Additional Roles for RAG Proteins

In vitro reactions using RAG-supplemented cell extracts demonstrate persistent association of RAG proteins with CEs and SEs following coupled cleavage. This complex contains additional proteins believed to be involved in the joining reaction *(2,106).* Many details of this complex remain to be defined, for example whether a single complex contains CEs and SEs, and how the(se) complex(es) is (are) remodeled over time. The importance of a stable post-cleavage complex is emphasized by observation that coding joint formation is facilitated when the cleavage and joining reactions are temporally linked *(146).* One role for RAG proteins in the post-cleavage complex may be the opening of hairpin-coding ends *(13).* Other potential roles of RAG proteins include the retention of DNA ends in close proximity to facilitate ligation, the protection of DNA termini from nucleases, and/or the regulation of subsequent steps of V(D)J recombination *(82,105,223).* For example, the post-cleavage complex may recruit DNA repair or signaling proteins *(45).* There is evidence for direct involvement of RAG proteins in facilitating the joining of coding and signal ends by other DNA repair proteins *(41,265,294).*

Recent studies support the idea that *Rag* genes may have evolved from an ancient transposase. Multiple mechanistic similarities between V(D)J recombination and transposition have been identified. In both reactions, a recombinase recognizes sequences that flank the mobile element, introduces a DSB which dissociates the signal sequences from the transposed element, and then cellular DNA repair machinery is co-opted to rejoin the DSB *(234).* Furthermore, the two *Rag* genes encoding the recombinase are closely linked, and found only in vertebrates in which antigen-receptor gene rearrangements generate antigen-specific immunity *(154).* This genomic arrangement and coincident evolutionary introduction of *Rag* genes and RSS elements suggest that both may have been introduced together by an ancestral transposon *(1,107,154,234,274,283).* Recent in vitro evidence shows that RAG proteins can act as a transposase, mediating cleavage and insertion of SEs into a target site *(1,107).* A molecular signature of retroviral integrases is left behind: short duplications of the target DNA immediately flanking the mobilized fragment *(1,107).* Currently there is no evidence that the V(D)J reaction causes integration of one piece of DNA into another in cells *(1,107).* The excitement generated by the in vitro evidence of transposase-like behavior ensures intensive future efforts to discover whether RAG proteins can catalyze other DNA transactions in lymphocytes. RAG-mediated transposition is a possible mechanism by which an antigen receptor locus could be misdirected to an unrelated genomic region, a frequent characteristic of chromosomal translocations isolated from lymphoid malignancies. Thus, the same mechanism that may have established the capacity for antigen-specific immunity in early vertebrates may have nefarious potential to compromise genomic stability.

4. GETTING IT TOGETHER: THE V(D)J JOINING REACTION

Following RAG1/2-mediated cleavage at RSS, two types of ends must be rejoined, blunt SEs and hairpin CEs. SEs are ligated directly without the addition or deletion of any nucleotides. CEs are processed, both to open the hairpin and to add or delete nucleotides, before ligation. This nonconservative end-joining process is similar to the process of nonhomologous end joining (NHEJ). NHEJ is a major DSB repair pathway in mammalian cells and functions to a lesser extent in yeast *(114).* V(D)J recombination

is a unique form of NHEJ because nontemplated nucleotides can be added at the coding joints, creating junctional diversity that vastly increases the antigen-receptor repertoire.

4.1 Coding End Processing and Joining

The first step in CE processing is the opening of the hairpin ends produced by RAG1/2-mediated cleavage. The endonuclease activity responsible for hairpin opening has not been identified. However, recent work has shown that RAG1/2 can nick hairpins in vitro as part of a post-cleavage complex *(13).* Hairpin opening is dependent on the DNA-dependent protein kinase (DNA-PK; Subheading 4.1.2.) because hairpin ends accumulate in lymphoid cells of both SCID *(73,235)* and *Ku80*$^{-/-}$ mice *(309).* However, SCID cells can nick and ligate transfected hairpin substrates, suggesting that the putative role of DNA-PK in hairpin opening differs for extrachromosomal and chromosomal substrates *(150).*

Addition of nontemplated nucleotides (N-regions) at coding joints is catalyzed by the lymphoid-specific terminal deoxynucleotidyl transferase (TdT) prior to coding joint ligation *(78,132).* Although TdT contributes to the generation of diversity, it is dispensable for V(D)J joining itself *(76,82).* Recent work has shown that nucleotide addition and loss at the CE may be influenced by sequence motifs within the coding flanks *(192).* Removal of nucleotides is not completely random *(247)* and may result from the nicking on both sides of the hairpin *(192).* The addition of self-complimentary or palindromic (P) nucleotides results from the asymmetric opening of hairpin CEs. Before joining, CEs may be aligned by short regions of microhomology *(149).* One could imagine a dynamic equilibrium at the CE, with TdT activity lengthening the DNA ends and an exonuclease shortening the ends. Because microhomology-mediated recombination may involve as little as a single bp match *(205),* TdT activity could increase the probability of creating microhomology. Thus, N nucleotide addition may function both to increase diversity of variable regions and to facilitate V(D)J joining.

Identification of the proteins involved in SE and CE joining during V(D)J recombination was propelled by studies of X-ray cross complementation mutants *XRCC4 – XRCC7,* cell lines bearing DNA repair-deficiencies that were subsequently shown to have defects in V(D)J recombination (Table 1). Several ubiquitously expressed genes responsible for the radiosensitivity and V(D)J defects have been identified. They are the DNA-PK (XRCC7), the autoantigens Ku70 and Ku80 (XRCC5), and XRCC4, which interacts with DNA ligase IV the enzyme that joins coding and signal ends *(43,85).*

4.1.1. Biochemical Characterization of DNA-PK$_{cs}$

DNA-PK$_{cs}$ was recognized as a kinase activated by free double-strand DNA (dsDNA) ends in human cell extracts *(29,144).* Initial biochemical purification revealed that this kinase activity co-purified with three components: a heterodimer of Ku70/Ku80 and the catalytic subunit called DNA-PK$_{cs}$ *(34).* DNA-PK$_{cs}$ activity was identified in a wide range of organisms, including mouse, hamster, *Xenopus,* and *Drosophila (64),* and homologs have now been cloned from humans *(19,39,94,214),* mice *(6,68),* and horses *(252).* Partial cDNAs have also been isolated from hamster *(19)* and *Xenopus (136).* DNA-PK$_{cs}$ belongs to a family of serine-threonine kinases involved in DNA damage surveillance and repair that are related to phosphatidyl inositol-3-kinase (PI3-kinase; *94).*

Table 1
V(D)J Recombination on ECRS Assays in DSB Repair Mutants

		Signal joints		Coding joints		
Species	Mutant/parent cell line	Relative frequency[a]	Fidelity %[b]	Relative frequency[a]	Mutation	References
Mouse	SX9/SR-1	0.10	12	0.03	DNA-PK Leu_{3191}→Pro	*(69)*
Mouse	SCID	0.1~1.0	50~80	<0.01	DNA-PK Tyr_{4046}→Stop	*(6,17,19,48,162)*
Mouse	*DNA-PK*$^{N/N}$ ES line	0.29~0.86	100	0.004~0.02	DNA-PK knockout	*(73)*
Hamster	V3/AA8.4	0.22~0.54	70	0.03	DNA-PK, not identified	*(19,215,269)*
Hamster	*irs*-20/CHO-10B2	0.12	80	0.03	DNA-PK, Glu_{4120}→Lys	*(215)*
Hamster	XR-C1/CHO9	0.01~0.03	n.d.	0.01~0.44	DNA-PK, not identified	*(60)*
Horse	SCID	n.d.	n.d.	n.d.	DNA-PK, 5-bp deletion frame shift	*(252,300)*
Human	MO59J/MO59K[c]	1.0	95	<0.001	DNA-PK, not identified	*(133,145)*
Hamster	*xrs-6*/CHO-K1	0.05	0	0.02	*Ku80,* 13-bp insertion frame shift	*(188,255,270)*
Hamster	XR-V15B/V79	<0.01	n.d.	<0.02	KU80, deletion codons 317–417	*(61)*
Mouse	*Ku70*$^{-/-}$ KO cell line[d]	0.11	0	<0.004	*Ku70* knockout	*(86)*

[a] As compared to parental or wild-type cell lines.
[b] Percentage of signal joints formed that could be cut with ApaL 1.
[c] MO59J/MO59K are independent cell lines derived from a human glioblastoma. MO59J has no detectable DNA-PK activity, whereas MO59K does.
[d] Mouse-embryo fibroblasts with *Ku70* targeted disruption.

DNA-PK$_{cs}$ is activated by DNA in the absence of Ku, but Ku increases the selectivity of DNA-PK$_{cs}$ for dsDNA ends and stabilizes DNA binding by DNA-PK$_{cs}$ *(90,296,305).* This stabilization increases DNA-PK activity *(90).* These data led to a model in which Ku binds dsDNA ends and recruits DNA-PK$_{cs}$ to these sites. Ku then translocates along the DNA allowing DNA-PK$_{cs}$ to contact the dsDNA end, thereby activating the kinase. Blocking Ku translocation causes Ku and DNA-PK$_{cs}$ to remain in an enzymatically nonproductive complex *(90,296).* This model accounts for the inability of DNA nicks, hairpins, and cisplatin-damaged DNA to activate DNA-PK: even though Ku can bind to these discontinuities, it fails to expose dsDNA ends to recruit and/or activate DNA-PK$_{cs}$ *(90).*

4.1.2. On the SCIDs: DNA-PK$_{cs}$ Mutants

The expression of *DNA-PK$_{cs}$* rescues the radiation sensitivity and V(D)J recombination defects of SCID and hamster *XRCC7* mutant cell lines proving the genetic identity of these defects *(127,134,294,305).* The SCID mutation was identified as a nonsense mutation at codon 4046, resulting in the deletion of the C-terminal 83 amino acids *(6,19,48),* a region highly conserved among proteins involved in DNA damage surveillance *(48).* Although the murine SCID phenotype proved to be as radiosensitive as mutants defective in either Ku subunit or XRCC4, the V(D)J phenotype affected primarily coding joint formation (Table 1). In contrast, the equine SCID and targeted *Ku$^{-/-}$* mutations severely affected both coding- and signal-joint formation *(300).* Both murine and equine SCID cell lines were shown to lack detectable DNA-PK activity, while retaining normal levels of Ku DNA end-binding activity by direct measure *(300)* or complementation *(48,127,271).* The phenotypic discrepancy between murine and equine SCID and between murine SCID and Ku-deficient cells led several groups to hypothesize that the murine SCID DNA-PK retained some residual activity allowing the generation of near-normal signal joints.

This hypothesis was supported by the characterization of three additional mutant cell lines (Table 1). The murine cell line SX9 and the hamster cell line *irs-20* were found to contain *DNA-PK$_{cs}$* point mutations. These cell lines, along with another hamster cell line defective in DNA-PK$_{cs}$, XR-C1, had profound defects in both signal- and coding-joint formation. Given this evidence, it was suggested that the SCID DNA-PK$_{cs}$ mutation allowed the production of V(D)J signal joints and rare coding joints, the latter of which explains the "leaky" development of low numbers of mature T and B cells in aged SCID mice *(22,49).* However, human DNA-PK$_{cs}$-deficient cell lines could not mediate coding-joint formation on ECRS, despite apparently normal signal-joint formation *(133).* One interpretation of these seemingly paradoxical data was that the defective signal-joint formation in SX9 and *irs-20* cells resulted from a dominant negative effect of mutant DNA-PK$_{cs}$. If this is true, this dominant negative effect must be reversible by wild-type DNA-PK$_{cs}$ because both radiosensitivity and V(D)J recombination phenotypes were complemented by somatic-cell fusions with wild-type cells or by transfection of wild-type *DNA-PK$_{cs}$ (69,127,215,269,294).*

This debate can now be resolved, at least for mice. Gao et al. *(73)* generated a targeted knockout of the murine *DNA-PK$_{cs}$* gene by insertion of a *neo^{r}* cassette in exon 6, antisense to DNA-PK$_{cs}$. These mice, *DNA-PK$^{N/N}$*, have V(D)J recombination and lymphocyte development defects very much like that of SCID mice. In contrast, a mouse with a deletion of the DNA-PK$_{cs}$ kinase domain *(DNA-PK$^{-/-}$)* has a significant population of CD4+ CD8+ T cells *(268).* In *DNA-PK$^{N/N}$* embryonic stem (ES) cells, normal,

precise signal joints are detected on ECRS under conditions where coding-joint formation is almost completely abrogated. Similarly, normal TCR-δ signal joints were detected in the *DNA-PK*$^{-/-}$ mouse, although some D-J_H coding joints were detected *(268).* These differences may be owing to the different knockout constructs used. Analysis of the products of RAG1/2-mediated cleavage in SCID, *DNA-PK* $^{N/N}$, and *DNA-PK*$^{-/-}$ thymocytes showed that although SEs are normal *(73,268),* CEs accumulate as hairpin structures *(73,235).* Taken together, these data are consistent with a role for DNA-PK in the formation of coding, but not signal, joints.

Although both *DNA-PK*$^{N/N}$ and *DNA-PK* $^{-/-}$ mouse embryo fibroblasts are radiosensitive *(73,268),* the *DNA-PK*$^{N/N}$ ES cells were not *(73),* suggesting the presence of a DNA-PK-independent DSB-repair pathway that is not present in more differentiated cells. Similarly, cell fusions made between two SCID-like cell lines, $SCGR_{II}$xV3 or V3xV3, still manifest defective coding-joint formation, but are partially complemented for radiosensitivity *(269),* again invoking a DNA-PK-independent DSB-repair pathway. Perhaps this pathway involves homologous recombination such that in tetraploid fused cells the extra homologous templates facilitate this usually inefficient mode of DSB repair. It has also been shown that homologous recombination is stimulated by DSBs and may be more active in ES cells than in more differentiated cells *(55,158,228,259).*

4.1.3. Biochemical Characterization of Ku

Ku was initially described as a heterodimer of 70 kDa and 80 kDa protein subunits with DNA end-binding activity. Ku is able to translocate to internal sites on the DNA in a sequence-and ATP-independent manner *(50,307).* DNA-PK activity may regulate this translocation during end joining *(25).* DNA-dependent ATPase and nucleotide-dependent helicase activities for Ku have also been described *(27,279).* As with DNA-PK_{cs}, understanding of the role played by Ku in DSB repair came from studies of mutant cell lines *(224).* Mutations in *Ku80* were shown to be responsible for both the radiosensitivity and V(D)J recombination deficiency of the *XRCC5* complementation group *(59,61,188,255).* Although Ku alone does not have any kinase activity *(90),* it is a DNA-PK substrate in vitro *(33).* Phosphorylation of Ku and autophosphorylation of DNA-PK_{cs} in vitro causes the dissociation of the DNA-PK complex from dsDNA ends and cessation of DNA-PK kinase activity, although the in vivo significance of these phosphorylation events is unclear *(33).* Neither the ATPase nor the helicase functions of Ku are essential for DSB repair because site-directed mutagenesis of key residues in these motifs does not affect the ability of Ku80 to rescue *xrs-6* radiosensitivity. Furthermore, mutagenesis of some of the serine residues phosphorylated by DNA-PK also does not affect Ku80 rescue of *xrs-6* radiosensitivity *(255).* Several studies have demonstrated that DNA-PK_{cs} and Ku can simultaneously bind and bridge two DNA molecules *(30,203,221,305).* In addition, Ku transferred from one DNA molecule to another when the DNA fragments had complementary ends as short as 4 bp in length *(18),* and could activate DNA ligase activity under some conditions *(221).* These data are consistent with a model where Ku, DNA-PK_{cs} and/or DNA-PK function as scaffolding components during DSB repair.

Further evidence for DNA-PK_{cs} and Ku function in V(D)J recombination comes from studies of in vitro V(D)J recombination reactions. Cortes and her colleagues recapitulated signal- and coding-joint formation in separate reactions *(41,294).* In vitro sig-

nal-joint formation required the presence of core RAG1/2 proteins and cell nuclear extracts. These signal joints were precise and the dependence on nuclear extracts could be relieved by addition of T4 DNA ligase *(41,223).* Immunodepletion of the nuclear extracts with anti-Ku antibodies abolished signal joint formation, confirming the importance of Ku in this process. Coding joint formation required RAG1/2 and a fraction of HeLa cell nuclear extract that contained Ku70, Ku80, DNA-PK_{cs}, and a ligase *(294).* Furthermore, immunodepletion of extract with anti-DNA-PK_{cs} antibodies abolished coding-joint formation, while immunodepletion using anti-Ku antibodies only decreased coding-joint formation by 50%. These data are consistent with a primary function of Ku in chromatin remodeling at CEs, service as a SE bridge, or in stabilizing DNA-PK activity at the post-cleavage complex. If Ku-independent DNA-PK_{cs} activity is sufficient to mediate coding-joint formation, in vitro coding-joint formation would not be completely abrogated by immunodepletion of Ku. Therefore, although Ku, DNA-PK_{cs} and ligase activities are all essential for V(D)J recombination, Ku probably has functions in V(D)J recombination independent of its role in DNA-PK activation. It seems likely that future in vivo studies will reveal additional players recessed from the ends of DNA, perhaps involved in chromatin accessibility and remodeling.

4.1.4. You Don't Know What You've Got 'Til It's Gone: Ku Mutants

Cell lines with mutations in *Ku80* show defective coding- and signal-joint formation (Table 1), unlike the murine SCID defect, which primarily affects coding-joint formation. Studies using endonuclease-generated DSB in *xrs-6* cells showed that Ku was needed for the NHEJ of chromosomal DNA ends but not homologous recombination-mediated repair *(159).* Ku function is specific to the joining phase of V(D)J recombination, and is not required to protect SEs from nuclease degradation *(91).*

The generation of Ku80- and Ku70-deficient mice has allowed for a careful comparison of these phenotypes with SCID mice. In dramatic contrast to DNA-PK_{cs} mutations, *Ku80*$^{-/-}$ and *Ku70*$^{-/-}$ mice are proportional dwarfs as well as being lymphocyte-deficient *(86,197,202,309).* Expression of both Ku proteins is compromised in either single mutant, consistent with previous findings that co-expression is essential for the stability of each protein *(37,59,61,86,202,255).* In both types of Ku-deficient mice, B-cell development is arrested at the pro-B cell stage, probably because the rearrangement of Ig genes is severely compromised *(86,197,202,309).* SEs and CEs are full length in immature Ku$^{-/-}$ lymphocytes, and when present, V(D)J joints are reminiscent of those seen in SCID mice *(86,309).*

Although the stunted growth, radiosensitivity, and B-cell development phenotypes are very similar between *Ku70* and *Ku80* knockout mice, T-cell development shows some surprising differences, possibly indicative of subunit-specific Ku functions. In *Ku80*$^{-/-}$ mice, >90% of thymocytes are arrested at the CD4- CD8- DN stage and successful TCR rearrangement is rare *(197,309). Ku70*$^{-/-}$ mice, however, have a significant population of CD4+ CD8– and CD4– CD8+ TCR-β+ T cells in the thymus and secondary lymphoid organs *(86,202).* Further, *Ku70*$^{-/-}$ mice die of either intestinal neuronal disorders or of T-cell lymphomas, often of a CD4+ CD8+ TCR-β+ phenotype *(155).* Primary mouse *Ku70*$^{-/-}$ fibroblasts show multiple hallmarks of genomic instability: increased rate of sister chromatid exchange, radiosensitivity, increased rate of in vitro transformation, and 100% tumor growth in adoptive transfers to nude mice *(155).*

The authors suggested that Ku70 is a tumor suppressor in T cells, and proposed that the induction of a secondary DSB-repair pathway rescues thymocyte development in *Ku70*$^{-/-}$ mice. In the context of an inherent DSB-repair defect, developing *Ku70*$^{-/-}$ thymocytes are susceptible to oncogenic transformation. At present it is difficult to harmonize the phenotypic outcomes of mutations in DNA-PK, Ku80, and Ku70 with a simple model of their functions in V(D)J recombination. Rather, it will likely emerge that these proteins subserve both cooperative and independent functions in DSB repair, DNA replication, and transcription.

4.1.5. Biochemical Characterization of XRCC4 and DNA Ligase IV

XRCC4 was identified by complementation of the radiosensitive and V(D)J recombination-defective phenotypes of XR-1 cells by a human cDNA. However, sequence analysis of XRCC4 revealed no similarity to known proteins *(157).* The XRCC4 mutation in XR-1 cells reduces coding- and signal-joint formation in ECRS assays by at least 100-fold, with the rare coding joints formed resembling microhomology-directed events seen in SCID cells *(92,157,271).* At a biochemical level, XRCC4 is a disulfide-linked homodimer or multimer that can complex with DNA, Ku, and DNA-PK in vitro *(141).* DNA-PK phosphorylation of XRCC4 in vitro can abrogate XRCC4 DNA binding *(189).* Although XRCC4 is an effective substrate for DNA-PK and other kinases *(43,141),* the phosphorylation sites are dispensable for DNA repair, and may not be physiologically relevant in this context *(141).*

DNA ligase IV was identified as an XRCC4-interacting protein by virtue of both co-purification and co-immunoprecipitation *(43,83).* XRCC4 was shown to be a potent activator of DNA ligase IV activity both in transfected cell lines and in vitro *(83,189)* and stabilizes DNA ligase IV protein levels in XR-1 cell lines *(24).* Further, only those XRCC4 deletion mutants that interact with and activate DNA ligase IV rescue V(D)J recombination in *XRCC4* mutant cells *(85).* In contrast, V(D)J recombination is normal in cells deficient in DNA ligase I and DNA ligase II/III *(109,211,276).* These data led to the hypothesis that DNA ligase IV, in association with its activator, XRCC4, is the ligase used in V(D)J recombination. This hypothesis has been confirmed in both knockout cell lines and mice (Subheading 4.1.6.)

4.1.6. Unexpected Links between Immune and Nervous Systems: XRCC4 and DNA Ligase IV Mutants

V(D)J recombination and DSB repair are abolished in a human pre-B cell line disrupted at both alleles of DNA ligase IV *(84)* and in cells from DNA ligase IV knockout mice (*LigIV*$^{-/-}$; *65).* Rescue of V(D)J recombination in these mutant cells can be effected by DNA ligase IV, but not by DNA ligases I or II/III *(84).* Interactions between DNA ligase IV and XRCC4 are crucial, as mutations that abrogate their contact blocked this rescue *(84).* Examination of fetal lymphoid tissues in *LigIV*$^{-/-}$ or *XRCC4*$^{-/-}$ mice revealed an arrest of T- and B-lymphocyte development at the pro-T and pro-B cell stages (Fig. 2; *65,74).* Overall lymphoid-precursor cell number is decreased, perhaps owing to death of progenitors that initiate, but cannot complete, V(D)J recombination. The rare coding joints detected in *XRCC4*$^{-/-}$ mice have large deletions reminiscent of those seen in SCID mice *(74).* Taken together, these data demonstrate that DNA ligase IV, in association with XRCC4, is essential for both V(D)J recombination and DSB repair, but do not rule out the possibility that other ligases might also be involved *(65).*

Interestingly, *LigIV*$^{-/-}$ and *XRCC4*$^{-/-}$ knockouts cause early embryonic lethality. Histological examination of the embryos reveals a severe defect in neurological development resulting from increased cell death *(74).* These observations led to the examination of neuronal development in other NHEJ mutant mice. Gu et al. demonstrated increased neuronal apoptosis in *Ku70*$^{-/-}$ and *Ku80*$^{-/-}$ embryos, but not in *DNA-PK*$^{-/-}$ embryos *(86a).* In all cases examined, the onset and peak of neuronal apoptosis correlates with neuronal development. Furthermore, the degree of neuronal apoptosis is inversely related to the degree of leakiness in signal joint formation in ECRS assays in mutant MEFs. While the roles of these proteins in neurological development are unknown, it is intriguing that these phenotypes provide another link between neurological defects and DSB repair. Additionally, 25% of *Ku70*$^{-/-}$ knockout mice die as a result of intestinal neuron degeneration *(155).* Patients with mutations in the DNA damage surveillance gene, ataxia telangiectasia mutated (*atm;* Section 8.4.), or with mutations in p95, a component of the Mre11/Rad50 complex (*See* Section 5.), also show neurological defects. These observations are suggestive of an as yet undefined, but essential, role for DNA repair in neurological development.

Two alternate, though not exclusive, hypotheses have been proposed for the role of DNA repair in neurological development *(37a,73a,86a,143a).* The first invokes targeted DNA rearrangements as an essential part of neuronal development, perhaps responsible for the generation of neuronal diversity. The second suggests that developing neurons are exquisitely sensitive to DNA damage and undergo apoptosis as a consequence to perturbations in the mechanisms of DNA repair. The source of DNA damage could arise from site-specific cleavage during development or be the result of normal cellular processes, such as replication or oxidative metabolism. While searches for a potential target of neuronal DNA rearrangement have not yet yielded fruit (*86a,* unpublished data), recent genetic evidence suggests that neuronal lethality is the result of the cellular response to unrepaired DNA as opposed to defective NHEJ per se *(73a).* The lethality of *XRCC4*$^{-/-}$ and *LigIV*$^{-/-}$ can be rescued by mutations in the DNA damage checkpoint involving ATM and p53 (Sections 8.3. and 8.4.). Neither *ATM*$^{-/-}$ *LigIV*$^{-/-}$ *(143a)* nor *p53*$^{-/-}$ *XRCC4*$^{-/-}$ *(73a)* embryos show increased neuronal apoptosis, and mice of both genotypes survive to birth with morphologically normal CNS. However, *ATM*$^{-/-}$ *LigIV*$^{-/-}$ mice die two days postnatally, whereas *p53*$^{-/-}$ *XRCC4*$^{-/-}$ mice succumb to pro-B cell lymphoma at 6 weeks of age. Neither genotype rescues V(D)J recombination defects, but the *p53*$^{-/-}$ *XRCC4*$^{-/-}$ pro-B cell lymphoma have translocations between the IgH locus and the c-*myc* oncogene *(73a,143a).* The early postnatal death of ATM-deficient *LigIV*$^{-/-}$ mice suggests that other organ systems may be affected or that the CNS may not be entirely functional, despite the abrogation of postmitotic neuronal apoptosis. These data support the hypothesis that an alternative DNA repair process functions in cells with unreapired DNA damage that are not eliminated by the p53-dependent apoptotic pathway.

5. LESSONS FROM YEAST NHEJ

The studies previously discussed have revealed many similarities between V(D)J recombination and NHEJ, both with respect to the genes involved (Table 1) and the DNA joints produced *(114,150).* It has been well-established that the yeast *S. cerevisiae* repairs DSBs primarily by homologous recombination, involving members of the

RAD52 epistasis group (reviewed in *114,118*) and is discussed further in Chapter 16 in Vol. I. However, the identification of the yeast Ku homologs, *HDF1* (Ku70;)*(62)* and *HDF2* (Ku80; *186)* revealed a second repair pathway with striking parallels to mammalian NHEJ (reviewed in *277*). The information garnered from studies with yeast provide us with a road map for the exploration of the proteins that catalyze NHEJ and by analogy, the joining of V, D, and J ends during antigen receptor gene rearrangement.

In addition to NHEJ, Ku has multiple functions in genome maintenance in yeast. These include essential roles in telomere silencing, length maintenance, structure and subnuclear organization *(23,80,137,196).* Other genes involved in chromatin silencing and telomere-length maintenance, (*MRE11, RAD50,* and *XRS2*), also function in Ku-dependent NHEJ, possibly as an exonuclease *(23,196,278),* although a recent report suggests nuclease-independent functions in NHEJ *(190).* Human MRE11 and RAD50 homologues have been identified *(53,212).* p95 is encoded by the gene mutated in Nijmegen breakage syndrome (NBS), a human disorder characterized by mental retardation, cellular radiosensitivity, chromosomal instability, early-onset lymphoma, agammaglobulinaemia, and mild to moderate lymphopenia *(116,280).* Detection of p95 as the NBS protein relied in part on the complex formed with human RAD50 and MRE11 in vivo *(28,175,285).* This complex can unwind dsDNA and cleave hairpin DNA *(206).* The genetic tools available for yeast have been powerful in elucidating the roles of shared players in NHEJ in yeast and mammals. It is important to note that although sequence and function are conserved among some proteins (e.g., Ku70/80) involved in NHEJ across species, function is not always conserved (e.g., *MRE11,* by noncomplementation), and sequence is not conserved among others (e.g., *XRS2,* p95/NBS1). Moreover, no member of the *RAD53/MEC1*/DNA-PK_{cs} PI3-kinase family has been implicated in yeast NHEJ beyond the DNA damage detection stage. This pattern of shared and distinct proteins is consistent with unique participants in NHEJ in response to pathological vs physiological DNA damage, and for species-specific components.

6. TOWARDS A MODEL OF V(D)J JOINING

Although far from complete, the data support an emerging model for DNA-PK, Ku70/80, XRCC4, and DNA ligase IV function in V(D)J recombination. An early step may be Ku recruitment of DNA-PK to SEs at the post-cleavage complex (Fig. 4B). SEs could be the major activators of DNA-PK because hairpin-ended DNA does not appear to activate DNA-PK activity in vitro *(258).* Translocation of Ku along the DNA may then activate DNA-PK. The accumulation of hairpin ends in SCID and DNA-$PK^{N/N}$ mice *(73,235)* suggests that DNA-PK function is required for hairpin nicking in this chromosomal context. DNA-PK may enable RAG1/2 to nick the hairpins, remodel the post-cleavage complex, allow access of TdT and other joining factors, and/or facilitate CE release from the post-cleavage complex. Although the model in Fig. 4 shows coding-joint formation occurring within the post-cleavage complex, there is no direct evidence that this occurs. By analogy to Ku function at yeast telomeres, Ku could remodel chromatin to expose the CE for processing, and/or to align the ends for joining (Fig. 4C). Subsequent to hairpin opening, CE processing occurs. A dynamic equilibrium between the action of TdT and exonuclease activity may exist to produce short regions of homology at CEs facilitating pairing prior to coding-joint formation. A DNA polymerase would fill in any gaps, perhaps by an error-prone mechanism to increase diver-

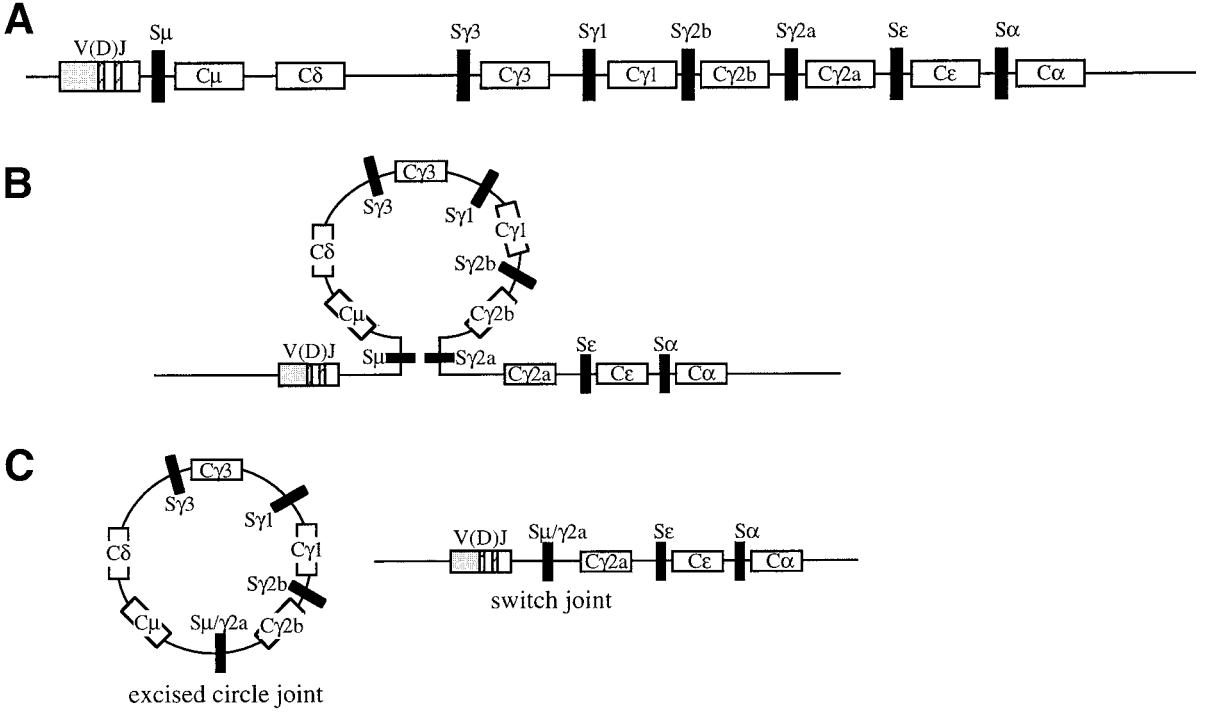


Fig. 5. CSR at the mouse heavy-chain locus. **(A)** S regions are present 5′ of each C_H gene, except Cδ. **(B)** Switch recombination is a multistep process. It likely involves the synapsis of the participating S regions followed by the introduction of DSBs in each S region. **(C)** Subsequent DNA repair juxtaposes the heavy-chain constant region of the switched gene and the V(D)J gene region at the switch joint. The intervening DNA is deleted from the chromosome as a circle. Drawing is not to scale and not all V, D, and J segments, or constant region exons, are shown.

sity *(111).* The large size of DNA-PK, the majority of which has no known function, makes it an attractive scaffolding protein. XRCC4 has been shown to interact with DNA-PK and Ku on DNA *(141),* so XRCC4 might recruit and activate DNA ligase IV to ligate CEs (Fig. 4D).

Although it is clear that DNA-PK function is not essential for signal-joint formation, studies of both DNA-PK mutant cells lines and mice has led to the hypothesis that DNA-PK function improves the frequency and fidelity of signal joints *(20).* DNA-PK function, in addition to being required for the opening of hairpin CEs, may facilitate signal-joint formation by recruitment of XRCC4/DNA ligase IV to the SE. DNA-PK autophosphorylation after end-joining may trigger disassembly of DNA-PK/Ku/DNA complexes *(33)* and the entire joining complex (Fig. 4E). Viewed from this perspective, V(D)J recombination is a carefully regulated process requiring coordinated activity of several proteins converged at dsDNA ends. Regulation of these steps probably involves control of both protein function (e.g., cleavage vs hairpin nicking by RAG1/2) and access to the V(D)J ends. Although several key players have been identified, their precise roles in V(D)J recombination remain enigmatic. Over the next several years, in vivo and in vitro experiments should provide a comprehensive picture of the molecular mechanisms of V(D)J recombination.

7. MAKING A GOOD THING BETTER: *DIVERSIFICATION OF ANTIGEN SPECIFICITY AND EFFECTOR FUNCTION IN MATURE B LYMPHOCYTES*

The antigen-binding specificity of B cells is not fixed once functional IgH and IgL rearrangements are completed in B-cell progenitors. IgV_H and IgV_L regions, as well as IgC_H regions can be altered at later times in a B cell's life-span. These alterations occur by a variety of mechanisms that involve targeted (but not site-specific) introduction of DSBs, though not all are rearrangement processes. Interestingly, these diversification events take place in a specialized microenvironment, called the germinal center (GC), which develops in secondary lymphoid organs, such as spleen, lymph node, and Peyer's patches *(216).* Prior to antigenic exposure, B cells reside in the primary follicles of lymphoid organs, whereas T cells reside in a distinct area. Following immunization, activated T and B cells migrate to the perimeter of the primary follicles where they interact to induce focal B-cell proliferation to generate GCs *(75).* GCs are the primary sites for three important modifications of Ig genes: hypermutation of V genes, receptor editing by V gene replacement, and class-switch recombination.

7.1. Somatic Hypermutation and Affinity Maturation

Somatic hypermutation is a process that refines V-region sequences in a template-independent fashion *(167,195,293).* The V-gene hypermutation rate of 10^{-3}–10^{-4} bp/cell division is 5–7 orders of magnitude higher than the spontaneous mutation rate. Unlike spontaneous alterations, hypermutation is selective for the 1–2 kbp surrounding a rearranged (but not germline) V_H or V_L gene. V-gene hypermutation exhibits a bias for base transitions *(15,195),* although insertions and deletions also occur *(301).* Some data suggest that purines in the coding strand are preferentially altered, but recent data suggest both strands are hypermutable *(56,187).* Finally, these mutations occur in a stepwise manner, such that a single B cell can accumulate multiple mutations during the many cell

divisions it undergoes in the GC. Most evidence suggests that TCR V genes do not undergo hypermutation, despite one study that claimed to find hypermutated TCR Vα regions in GC-derived cells *(308).* Though clearly an intriguing observation, this study did not involve purified T cells. Given that TCR genes are sometimes rearranged in B cells, it remains possible that GC B cells were the source of the mutated TCR Vα genes *(8).* In most mammalian species, hypermutation is an antigen-driven process operative in GC B cells. However, in sheep, hypermutation is an antigen-independent component of a developmental program used to diversify the primary Ig repertoire *(293).*

Comparisons of germline (unmutated) V_H/V_L gene sequences with the highly mutated ones derived from antigen-stimulated B cells reveal a significant clustering of mutations in the complementarity-determining regions (CDRs) that are crucial to antigen contact. Although mutational hotspots in the CDR appear to be intrinsic to the hypermutation mechanism *(15,195,218,306),* CDR clustering largely reflects profound selection for high-affinity antigen binding during the GC reaction. Once B cells cease proliferating in the GC, they express their modified BCR on the surface and migrate to the GC interior. This region also contains specialized antigen-presenting cells (APCs) whose surface is decorated with antigen-antibody complexes. B cells that can recognize antigen with high-affinity survive and re-enter the circulation as antibody-secreting plasma cells or long-lived memory B cells, and the remainder die by apoptosis *(172).* Thus, somatic hypermutation generates very high-affinity ($K_d = 10^{-8}$–10^{-9}) Ig from the low to moderate affinity ($K_d = 10^{-7}$) Ig produced in the primary repertoire. Strikingly, this affinity enhancement can be effected by as few as 3 amino acid substitutions. Although V gene hypermutation can be elicited under appropriate conditions in vitro *(117),* studies of mutant mice with defects in GC formation demonstrate that the specialized GC microenvironment is critical for affinity maturation in vivo *(40,71,122,173,174).* Interestingly, a subset of patients with common variable immune deficiency was reported to have defects in somatic hypermutation, identifying this process as critical for normal immune function *(148).*

The molecular mechanism of V-gene hypermutation remains mysterious, despite a recent torrent of studies in this area. An important clue was provided by studies implicating the transcriptional machinery in this process *(167,266).* Though hypermutation does not depend on an Ig promoter *(14),* sequences subjected to hypermutation are delimited by the promoter at the 5′ end of Ig loci. Strikingly, deletion of two IgL enhancers abolishes hypermutation without impacting expression of an IgL transgene, arguing that distinct *cis*-acting elements control Ig transcription and hypermutation *(14).* An IgH enhancer regulates hypermutation of V_H in a manner distinct from its regulation of transcription *(7).* Finally, hypermutation can be targeted to the IgC region by insertion of an IgL promoter immediately upstream *(210).* These data are consistent with the involvement of a transcription-coupled DNA repair process, such as excision repair, in V-gene hypermutation. Disappointingly, however, mice and humans with mutations in the excision repair genes *XPD* or *XPB* have normal V-gene hypermutation *(113,125,289).* Inactivating mutations of the *CSA, CSB,* or *XPA* excision repair genes also failed to debilitate hypermutation *(113,125,302).* Homologous recombination and base-excision repair are also not required for V-gene hypermutation, because this process is normal in mice with mutations in RAD54 or in 3-methyladenine-DNA glycosylase *(113).*

There has been substantial interest in a potential role for mismatch repair in somatic hypermutation. The mismatch repair pathway could facilitate hypermutation by processing transient DNA secondary structures in the V region. Alternatively, it could limit the extent of hypermutation by correcting mismatch mutations. Several groups reported that hypermutation following a primary immune response is normal or only slightly diminished in mice carrying a targeted mutation of the *Msh2* or *Pms2* mismatch repair genes *(12,66,113,213,302).* However, V genes from PMS2-deficient mice had significantly more adjacent base-pair substitutions *(302),* and *Msh2* mutant mice displayed more intense focusing of the mutations to the intrinsic mutational hot spots *(218),* than wild-type mice. Thus, rather than orchestrating hypermutation, the mismatch repair pathway may qualitatively alter the spectrum of mutations after they are introduced. However, hypermutation frequencies were diminished three to fivefold when Peyer's patch B cells from unimmunized mutant mice were examined *(66,218).* Even more surprising, hypermutation frequency was reduced 10-fold in PMS2-deficient mice *(31).* However, these mice also had very limited, quasi-monoclonal IgH and IgL V genes, causing tremendous selective pressure. In addition, the microsatellite instability associated with defective mismatch repair may be exacerbated during the extensive proliferation of antigen-activated B cells in the GC, and may lead to their premature death. A reduced life span of the mutant B cells could account for the paucity of hypermutated V genes accumulating large numbers of mutations, as well as their abnormal maturational status *(66,218,288).* Based on these observations, it was suggested that in contrast to most other cells, B cells may "fix" mismatches in the genome by using the newly synthesized strand of the V gene, rather than the parental strand, as the template for mismatch repair *(31).* Validation of this idea awaits further characterization of B-cell proliferative and maturational defects in mismatch-repair mutant mice, as these may indirectly affect hypermutation frequencies.

In summary, the molecular mechanism of V-gene hypermutation remains elusive, but recent studies are proving informative. Sale and Neuberger obtained evidence that DNA strand breaks are scattered within the mutation domain *(240).* Such breaks have been postulated but have been difficult to observe because, in contrast to RAG-mediated breaks, they would not likely be site-specific. To reveal the transient presence of DNA breaks during V-gene hypermutation, this group ectopically expressed TdT, reasoning that it would add nontemplated nucleotides to accessible 3′ ssDNA or dsDNA ends. The nature (ss vs ds) of the breaks and the mechanism by which they are induced remain obscure, but the authors propose a role for a DNA polymerase. Similarly, Bertocci et al. *(12)* suggest that hypermutation involves the recruitment of an error-prone DNA polymerase that mediates short-patch DNA synthesis occurring outside of global DNA replication. Whatever the mechanism, a potential role for dysregulated V-gene hypermutation in B-cell transformation and immune deficiencies provides continued incentive to define it *(79,148,185,250).*

7.2. Receptor Editing by V-Gene Replacement

Until recently, RAG expression was thought to be permanently extinguished once immature lymphocytes expressed functional antigen receptors. It is now clear RAG expression and V(D)J recombination can be re-elicited in Ig^+ B cells (reviewed in *199*). Self-antigen binding to immature B cells with high-affinity autoreactive BCRs induces

RAG-mediated V-gene replacement, generally with nonautoreactive V genes *(36,182,207).* This process presumably prevents the apoptotic elimination of self-reactive cells by editing their receptor specificity. Similarly, secondary rearrangements of TCR-α genes occur in immature T cells that fail to be positively selected by self-MHC, presumably to give them another chance to express a useful TCR *(290).*

More recently, RAG1 and RAG2 expression has been observed in antigen-activated B cells recovered from spleens and Peyer's patch GCs, and new V(D)J rearrangements were observed in these cells *(93,104,204).* The biological function of this renewed V(D)J activity is still unclear, given that it could generate autoreactive as well as useful B-cell clones. However, in contrast to receptor editing in immature B cells, editing in peripheral B lymphocytes is inhibited by antigen receptor cross-linking *(100,101,181),* suggesting this process is triggered only in GC B lymphocytes with diminished antigen-binding affinity. Not surprisingly, only B lymphocytes expressing low-affinity BCRs are able to induce a new round of V(D)J recombination *(100),* resulting in a drive towards improved antigen-receptor affinity. The locus organization of BCR genes suggests that these improvements occur primarily at the IgL locus as simple joining of two alternate V and J regions during each round of V(D)J recombination. New V(D)J recombination at the IgH locus is impossible as the unused D segments are deleted during the V-to-DJ joining. However, V segment replacement, using an RSS-like sequence within the IgH coding region, has been documented *(35,130,226).* Mature T cells may also undergo receptor editing, but the reasons and regulation are not yet clear *(178).*

7.3. Class-Switch Recombination

Like V-gene hypermutation and some forms of receptor editing, class-switch recombination (CSR) is an antigen-dependent process that takes place in the specialized GC microenvironment. However, in this case, mature B cells change antibody-effector functions without altering antigen specificity (reviewed in *38,263*). The different antibody classes differ in their ability to fix complement, activate APCs and natural killer (NK) cells, and induce cytokine production. These functions are essential for efficient immune responses to foreign antigens and destruction of virally infected and transformed cells. Like V(D)J recombination, CSR involves the generation and repair of DSBs. Although the mechanism of CSR has not been defined, it involves intrachromosomal recombination between the switch (S) regions 5′ of the participating heavy chains (C_H; Fig. 5). S regions contain tandemly repeated segments of DNA, 1–10 kbp in length, located 5′ of each C_H gene, except Cδ. While V(D)J recombination occurs only at RSSs, class-switch events occur within the S regions of the switched partners, but without apparent sequence specificity *(263).* Switches involving Igμ can even occur outside of the Sμ *(38,142).* Another difference between V(D)J recombination and CSR is that although productive V(D)J rearrangement at IgH results in allelic exclusion, CSR occurs on both the productive and nonproductive C_H alleles *(263).*

The proteins involved in CSR have not been fully elucidated. CSR occurs ex vivo in pre-B cells from RAG2-deficient mice under appropriate stimuli, demonstrating that the RAG recombinase is not essential for CSR *(231).* Recently, a putative B-cell-specific switch recombinase activity was isolated from spleen-cell nuclear extract and identified as a multiprotein complex *(21).* One component of the complex, SWAP-70, was suggested to be a specific recruiting element, assembling the switch recombinase from universal compo-

nents, very similar to the process seen in V(D)J recombination. This study used extrachromosomal switch-recombination templates to assay switching, however the switched products were not sequenced. Confirmation of this complex as the elusive switch recombinase awaits confirmation of S-region specificity and genetic analyses of knockout mice.

Although switch recombinase activity is unique to B cells, evidence is mounting that, as with V(D)J recombination, the joining of switch regions depends on the general DNA repair components DNA-PK, Ku70, and Ku80 *(32,171,231).* CSR cannot be induced in ex vivo SCID pre-B cells, indicating an essential role for DNA-PK in CSR *(231).* Casellas et al. *(32)* used *Ku80*$^{-/-}$ mice with targeted rearranged VDJμ heavy and VJ_κ light chain transgenes to show that Ku80-deficient B cells could respond to switch signals by upregulating germline C_H transcripts and introducing DSBs into switch regions. However, these DSBs could not be resolved and neither mature Igγ1 transcripts nor Sμ-Sγ1 DNA recombination was detected. Using a similar Ig transgenic approach, Manis et al. *(171)* demonstrated that B cells from *Ku70*$^{-/-}$ mice respond to class-switch signals, but neither the DNA products of class switching nor serum IgG or IgE were detected. Experiments using targeted, rearranged VDJ heavy and VJ light-chain genes in a SCID background could determine whether DNA-PK function is required for processes other than V(D)J recombination and CSR. These could also determine if, as with V(D)J recombination, impaired SCID DNA-PK function affects the two ligation products of CSR asymmetrically, the switch joint vs the excised circle joint (Fig. 5C).

Several pathogenic consequences of defective CSR have been identified. The X-linked hyperimmunoglobulin M (X-HIM) syndrome is characterized by severely restricted antibody repertoire owing to a defect in the CD40 ligand gene that results in impaired T-cell function *(70).* This inherited immune deficiency causes recurrent infections and an increased susceptibility to B-cell lymphoproliferative disease. The latter may result from the absence of CD40 receptor engagement for the induction of Fas-mediated apoptosis of proliferating B-cells *(70).* Studies of multiple myeloma tumors and cell lines have indicated that chromosomal translocations involving the IgH switch regions are an early step in disease development *(11).* Burkitt's lymphoma in humans and murine plasmacytomas result from the translocation of c-*myc* to the IgH locus, likely during abnormal CSR *(217,251,272).* It is clear that careful regulation of CSR is necessary both for the development of an effective immune system and for the prevention of detrimental chromosomal translocations.

8. V(D)J GONE WRONG: *ONCOGENIC POTENTIAL OF V(D)J RECOMBINATION*

The DSBs generated by V(D)J recombination, as well as those resulting from genotoxic stress, are repaired by NHEJ *(257).* V(D)J misjoining events can transcriptionally activate oncogenes or inactivate tumor-suppressor genes, resulting in dysregulated cell growth and accumulation of mutations *(217).* Because development of lymphoid-cell precursors requires physiological DSBs coupled with bursts of proliferation, they may be especially vulnerable to the oncogenic consequences of such events. To minimize the occurrence of aberrant recombination events, RAG-generated DSBs must be placed precisely adjacent to target gene segments and the synaptic complex must promptly join the resulting gap. Poor fidelity in either process has serious consequences for the genomic stability of an organism.

8.1. Aberrant Rearrangements

Misdirected RAG activity during V(D)J rearrangement has been implicated in defects associated with unusual chromosomal rearrangements. Trans- or interlocus V(D)J rearrangements can occur in cells from normal mice and humans and may increase the immune repertoire *(129).* However, the presence of these aberrant receptor-gene rearrangements may herald genetic instability with potentially dangerous consequences. Indeed, translocations between proto-oncogenes and antigen receptor genes are a common feature of human lymphoid malignancies *(129,217),* where over 50% of the cases examined have a translocation between an antigen receptor and a growth-promoting gene (reviewed in *128*). The mechanism of these rearrangements is uncertain but it has been suggested that during V(D)J recombination, RAG proteins may catalyze a DSB at one legitimate RSS and at one "cryptic-signal sequence" at an unrelated chromosomal site *(107).* Cryptic-signal sequences resembling authentic RSSs are numerous in the genome and may be targets of RAG activity *(151).* Alternatively, chromosomal translocations may arise from defective alignment of the DNA strands before or after DNA cleavage at two authentic RSSs. The frequency of such aberrant chromosomal rearrangements is likely restricted by chromatin accessibility, limiting RAG proteins from binding sites not intended for V(D)J recombination. Ultimately, the potentially devastating effects of an aberrant recombination event are limited by cellular selection. Lymphocytes harboring "illegitimate" rearrangements would fail to be selected unless they also express the products of normal V(D)J rearrangements *(151).* However, cells in which a growth- or survival-promoting gene (e.g., a proto-oncogene) is activated by juxtaposition to the potent enhancers within the antigen-receptor locus may proliferate despite the absence of normal selection signals. Thus, uncoupling V(D)J recombination from normal cellular selection for functional antigen receptors puts lymphocytes at risk of neoplastic transformation into lymphoma or leukocytic leukemia *(217).* The consequences of putative mistargeting of RAG activity are restricted to lymphocytes by virtue of tissue-specific expression of these enzymes. In contrast, the components involved in V(D)J joining are ubiquitously expressed and have other DNA repair and regulatory functions in the cell.

8.2. Cell Cycle and V(D)J Recombination

The signaling pathways responsible for the mobilization of DNA repair systems are still being defined, both in terms of identity and temporal relationships of the component molecules. The response to DNA damage involves surveillance molecules (detectors) conveying signals that elicit appropriate cellular responses (effectors). These responses include cell-cycle arrest and activation of DNA repair, or programmed cell death (apoptosis). Cell-cycle arrest facilitates DNA repair preventing the replication of mutated DNA strands (G1-S transition) and the transmission of damaged chromosomes to daughter cells (G2-M transition). As was discussed previously, the initiation of V(D)J recombination is coordinated with the cell cycle, in part by restricting the accumulation RAG2 protein to G0/G1 *(51).* It is not surprising that the activity of certain DNA repair components is also the highest at this time *(77,110,143,157).* Similar to yeast and higher eukaryotic cells exposed to genotoxic agents, lymphoid progenitors undergoing V(D)J recombination are arrested in G0/G1 *(108,209),* presumably ensuring that V(D)J-specific DSBs are joined prior to the onset of S phase *(246).*

8.3. p53 and V(D)J Recombination

The *p53* tumor-suppressor gene encodes a tetrameric transcription factor that plays a central role in the response to signals from damaged DNA by activating genes involved in cell-cycle arrest and apoptosis *(131,298).* Basal levels of p53 are low in most cells *(63,201,225,230).* In response to DNA damage insults as discrete as a single DSB *(88,89,110,120,166,170,194),* p53 accumulates owing to post-transcriptional stabilization, localizes to the nucleus *(67),* and binds to specific DNA sequences to activate target genes *(275).* Illustrating the efficiency of DSB management during normal V(D)J recombination, p53 protein is undetectable in wild-type thymocytes actively engaged in TCR rearrangement *(88).* In addition, lymphocyte-compartments of p53-deficient mice are normal, indicating that p53 is not essential for the for V(D)J recombination *(54),* including the associated G1 arrest *(88).* However, p53 is vital for management of DNA damage as indicated by the development of aneuploidy in p53-deficient cells treated with DNA-damaging agents *(131).* This loss of genomic stability underlies the cancer-prone phenotypes of the inherited Li-Fraumeni cancer syndrome *(169)* and $p53^{-/-}$ mice *(54,112,139).* Seventy to ninety percent of the spontaneous tumors observed in p53-deficient mice are lymphomas, with a mean onset at 4–5 mo of age *(54,95,96,112).* This lymphoma susceptibility remains in the absence of V(D)J recombination, as $Rag^{-/-}p53^{-/-}$ double mutant mice also display a high incidence of thymic lymphoma *(161).* However, evidence for the protective role of p53 in the context of V(D)J recombination is evident in SCID mice where checkpoint and repair defects collide. SCID lymphoid precursors accumulate CEs *(235,310)* and these DSBs are sufficient to cause p53 upregulation in SCID thymocytes *(88).* Disruption of the p53 DNA-damage checkpoint in $p53^{-/-}$ SCID double mutant universally results in pro-lymphocyte aneuploidy and lymphoblastic leukemia with a mean onset at 2 mo of age *(88,191).* In addition to the p53 response to physiologic V(D)J breaks in SCID cells, p53 expression is also induced in response to γ-irradiation, indicating that stabilization and function of p53 is not dependent on wild-type DNA-PK *(88,191).* An attractive candidate gene for a role in p53 stabilization/activation was identified by studying patients with ataxia telangiectasia (AT), and subsequently mice with targeted *Atm* mutations, whose cellular response to DNA damage, including p53 induction, is defective *(121,123).*

8.4. ATM and V(D)J Recombination

AT is a rare, autosomal recessive disease characterized by progressive cerebellar ataxia and occulocutaneous telangiectases, immune deficiencies, increased predisposition to lymphoid malignancies, and extreme sensitivity to ionizing radiation (reviewed in *140,236*). In $Atm^{-/-}$ mice, the development of thymoma is dependent on the initiation of V(D)J recombination *(160).* Cellular abnormalities include cell-cycle defects, sensitivity to γ-irradiation, and an increased incidence of chromosomal translocations, all of which result in increased genetic instability. AT was mapped to mutation of the *ATM* gene. *ATM* encodes a 370 kDa protein, that along with DNA-PK, is part of the large superfamily of PI3-kinase related proteins *(242,243).* Despite identification of many spontaneous *ATM* mutations in AT patients, the exact role of this protein remains unclear. ATM may have a direct role in DNA repair *(115),* however, the kinetics of DNA repair are normal in ATM-deficient cells *(236).* Importantly, ATM may be a regulator of signal transduction in response to DNA damage, activating cell-cycle check-

points, DNA repair mechanisms, or apoptosis *(236).* ATM may also affect chromatin conformation, thereby regulating susceptibility to DNA damage as well as access of recombination/repair machinery to DNA lesions *(129).* It is now generally accepted that *ATM* mutations result in DNA damage checkpoint defects that account for the genomic instability in ATM-deficient cells *(115,236,273).*

ATM and p53 are likely co-participants in at least one pathway *(297)* in which the ATM-dependent dephosphorylation of p53 leads to its association with a 14-3-3 protein resulting in enhanced p53 stabilization *(291).* Additionally, recent evidence suggests that ATM directly phosphorylates p53 in response to ionizing radiation *(9,26,236).* Characteristic of the genetic instability associated with *ATM* mutations, AT patients' lymphocytes show increased chromosomal aberrations, many involving chromosomes 7 and 14 in the vicinity of TCR and Ig genes *(128,140).* Although a DNA recombination defect was first suspected, one study using ECRSs suggests that V(D)J recombination is normal in cells of AT patients *(109).* If so, the mechanism of immune deficiency in AT patients and $Atm^{-/-}$ mice is completely unclear. Developmental studies in ATM-deficient mice revealed phenotypic differences between their T- and B-lymphoid precursors and those of $Atm^{+/-}$ or wild-type controls *(10,58,184,303,304).* Most striking is the depletion of the more mature T-cell populations. However, neither a profound arrest in lymphocyte development nor a striking V(D)J recombination defect characteristic of SCID mice was observed. Given that p53-deficient mice do not show a deficit in either precursor or mature lymphocytes, the effect of ATM on lymphocyte development is likely p53-independent, perhaps affecting cellular proliferation or pre-senescent apoptosis. Thus, mechanistic connections between DNA-PK, p53, and ATM in lymphocyte development, V(D)J recombination, and the cellular response to DNA damage are likely, but not yet well-understood.

Ongoing inquiry into the regulation of V(D)J recombination will no doubt involve exploration of molecular pathways already identified to serve in the context of generalized DNA repair. The involvement of 14-3-3 proteins in the stabilization of p53 is reminiscent of a pathway involved in activation of cyclin-dependent kinase Cdc2, primarily examined in yeast models. In response to DNA damage, Cdc2 is inhibited by specific tyrosine phosphorylation resulting in a G2 arrest. The phosphorylation status of Cdc2 is maintained by kinases (wee1p and mik1p in *S. pombe*) and Cdc25 phosphatase (*S. pombe* and human; *227).* Cdc25 itself is phosphorylated by the Chk1 protein kinase *(241)* and subsequently sequestered in a complex with 14-3-3 *(208).* An interesting connection between Chk1 and p53 is suggested by the finding that cdc2 is involved in p53 phosphorylation *(16).* Indeed, Chk1 may be activated via phosphorylation by DNA damage-responsive protein kinases such as *S. pombe* rad3p, or human ATM or ATM-related protein, ATR. Mutations in DNA-PK_{cs} or Ku result in inefficient DSB repair and lead to impaired lymphocyte development (immunodeficiency) and genomic instability resulting in neoplastic transformation. The ability to detect DNA damage and engage appropriate signaling pathways is an important prerequisite for the mobilization of DNA repair proteins and cell-cycle arrest. Indeed, mutations in known DNA-damage detector and effector components, such as ATM and p53, result in profound tumor susceptibility, particularly in the lymphoid lineages. As yet, these molecules have not been implicated in V(D)J recombination, CSR, or somatic hypermutation.

9. CONCLUDING REMARKS

V(D)J recombination is a highly regulated process that couples successful DNA rearrangement with lymphocyte proliferation and maturation. The introduction of V(D)J-specific DSBs is regulated at two levels: through the lymphoid-specific expression of the RAG1/2 endonuclease and by control of chromatin accessibility to RAG1/2. Once cut, the DNA is rejoined using the general cellular DNA-repair machinery, involving DNA-PK_{cs}, Ku, XRCC4, and DNA ligase IV, although RAG1/2 may have specialized roles for V(D)J end processing. Further understanding of the process of NHEJ, in yeast and mammals, should facilitate understanding of V(D)J recombination.

The oncogenic potential of aberrant V(D)J recombination and CSR is evidenced by the high occurrence of translocations between antigen receptor and growth-promoting genes in human lymphoid malignancies. Additionally, the increased frequency of lymphocytic tumors in mice and humans with mutations in DNA repair and checkpoint genes, such as *Ku, DNA-PK, ATM, NB51,* and *p53,* demonstrate the importance of careful coordination of V(D)J recombination and cell-cycle regulation. Potentially oncogenic translocations may result from the (mis)targeting of the RAG1/2 endonuclease or perturbation of the DNA damage and cell-cycle checkpoints. V(D)J recombination provides a physiological context in which investigators can examine the complex mechanisms that maintain genomic stability.

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Interaction of Cell-Cycle Checkpoints with Muscle Differentiation

Troy Fiddler, Jing Huang, Elizabeth Ostermeyer, Teresa Johnson-Pais, and Mathew J. Thayer

1. INTRODUCTION

Damage to genomic DNA occurs spontaneously in all living cells, and represents a significant and constant problem. In addition, chemical or physical mutagens can cause a variety of DNA lesions, including base modifications, intra- and interstrand crosslinks and single- or double-strand breaks *(31)*. If left unrepaired, these DNA lesions can lead to mutations or loss of viability. Thus, cell-cycle checkpoints and DNA-repair mechanisms have evolved to ensure cellular survival in the face of DNA damage. Multicellular organisms have additional issues to deal with, including differentiation programs, as well as the existence of a limited number of stem cells required for renewal and repair of differentiated tissues. Because cell-cycle checkpoints and differentiation utilize the same key cell-cycle regulatory factors to mediate cell-cycle arrest, multicellular organism must integrate these two processes simultaneously. The subject of this chapter is the intersection of genetic alterations in the DNA damage-response pathway that interfere with overt differentiation.

2. CELL-CYCLE CONTROL BY ATM AND ATR

ATR is a mammalian gene with homology to *Schizosaccharomyces pombe rad3 (3a), Saccharomyces cerevisiae ESR1/MEC1 (77a), Drosophila mei-41 (39a),* and human ataxia telangiectasia mutated *(ATM) (46a). ATR* also shows homology to *FRAP/mTOR* and has been reported as *FRP1 (12a).* This recently identified family of proteins share common structural features, being large proteins (>2000 amino acids) with highly conserved carboxy-terminal kinase domains homologous to PI3-kinases. Mutations in the *ATM* gene lead to pleiotropic defects in cell-cycle regulation following DNA damage, including loss of the G1/S and G2/M checkpoints (reviewed in refs. *41a,42a*). Like ATM, ATR is a nuclear protein with protein kinase activity *(46a).* In the mouse, both ATM and ATR can directly associate with broken chromatin, as both proteins show complementary staining foci on meiosis I recombining chromosomes *(46a).* Recently, disruption of the *ATR* gene in mice has indicated that ATR functions early in develop-

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ment, as *ATR*$^{-/-}$ embryos die by day 7.5 of development *(8,18).* In culture, *ATR*$^{-/-}$ blastocyst cells display mitotic catastrophes and die of caspase-dependent apoptosis. Evidence that ATR has a DNA damage induced cell-cycle-checkpoint function come from analysis of kinase-inactive mutants of ATR (ATR-ki). Overexpression of ATR-ki has been shown to cause sensitivity to DNA-damaging agents and defects in cell-cycle checkpoints in response to ionizing radiation (IR) as well as ultraviolet (UV) light *(13,97).* Furthermore, overexpression of ATR-ki interferes with the late-phase Ser-15 phosphorylation of p53 in response to IR and to block UV-induced Ser-15 phosphorylation of p53 *(92).* One attractive interpretation of these results is that ATR-ki functions in a dominant-negative manner to interfere with the normal cell-cycle checkpoint functions of ATR. However, in the absence of information about ATR null cells exposed to DNA damage, it is difficult to determine whether ATR-ki is truly functioning as a dominant-negative, especially given that overexpression of wild-type ATR interferes with at least some p53 functions *(85).*

The *S. cerevisiae* Mec1p and the *S. pombe* rad3p proteins are involved in all of the known DNA-structure checkpoints in yeast (reviewed in ref. *11a*). In *S. cerevisiae,* there are two members of this large lipid kinase motif family, Mec1p and Tel1p. Mec1p has the highest degree of homology to ATR, and is an essential protein required for all the known DNA-structure checkpoints. The *TEL1* gene, which shows the highest degree of homology to ATM, is not an essential gene and does not show checkpoint defects when mutated. However, the double mutant *(mec1 tel1)* has increased radiation sensitivity. Furthermore, overexpression of *TEL1* can suppress the radiation sensitivity of *mec1* mutants. These results suggest a partial functional overlap between Mec1p and Tel1p in the control of DNA-damage-induced checkpoints. Taken together results suggest that, like Mec1p and Tel1p, ATM and ATR control cell-cycle checkpoints in mammalian cells.

3. CELL-CYCLE CHECKPOINTS AND p53

Mammalian cells with DNA-damage arrest in G1, S, and G2 phases of the cell cycle. In contrast, differentiating cells terminally arrest their cell cycles from the G1 phase of the cell cycle in a state known as G0. Primary among mammalian G1 checkpoint genes is p53. p53 is required for the G1 checkpoint, in which cells arrest in G1 in response to DNA-damaging agents such as IR *(46).* Utilizing cells from knockout mice, it has become clear that the p53 gene is critical for G1 arrest following γ-irradiation *(22).* In addition, p53 has been shown to be required for maintenance of the G2 arrest following IR *(9).* Following DNA damage, p53 protein levels are significantly increased in vertebrate cells grown in vitro *(16,46,51,53),* as well as in vivo *(38,57).* Although p53 mRNA levels do not change in response to DNA damage in many cell systems, the levels of p53 protein increases rapidly. The half-life of p53 protein increases substantially after DNA damage *(52,53,69),* and increased translation of p53 mRNA also contributes to p53 induction *(32,46,60).* The relative contributions of increased half-life and enhanced translation remain largely undefined. In addition to an increase in the levels of p53 protein, DNA damage is thought to result in activation of p53's ability to bind sequence-specific DNA and consequently transactivate gene expression *(83).* Furthermore, ATM has been implicated in regulation of p53, because cells from AT patients do not activate p53 normally in response to DNA damage (reviewed in ref. *24).* Activation

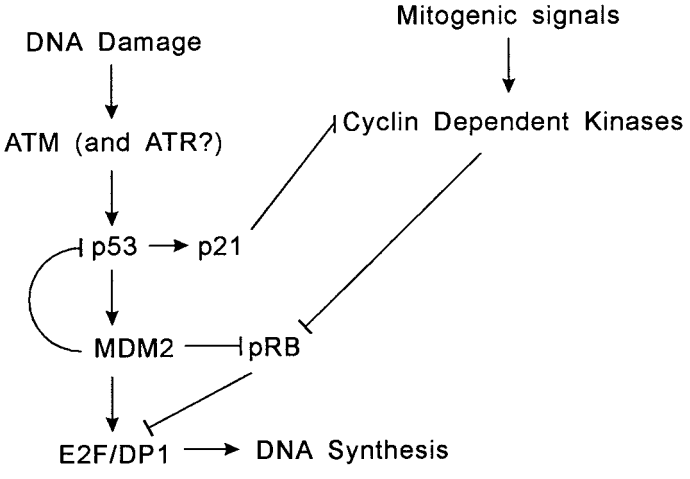


Fig. 1. Model for the G1 checkpoint.

of p53 DNA binding activity is thought to involve phosphorylation of serine 15 of p53 by ATM *(1,10)* and by ATR *(10).* In addition, dephosphorylation of p53 by an unknown phosphatase and subsequent association with 14-3-3 proteins leads to an increase in the affinity of p53 for sequence-specific DNA *(93).*

In response to IR, G1 arrest is mediated at least in part, through induction of the cyclin-dependent kinase (CDK) inhibitor p21/WAF1/Cip1 by p53 *(23).* p21/WAF1/Cip1 forms a complex with cyclin/CDKs, and represents one of the most studied p53 response genes *(25,40,47,78).* In normal cells, p21 is found associated with a variety of cyclin/CDK complexes, including the G1 cyclins CDK4/Cyclin D *(101,102).* In addition, p21 can inhibit the kinase activity of all of the cyclin/CDK complexes *(100).* A current model for the G1 checkpoint is illustrated in Fig. 1.

4. MUSCLE DIFFERENTIATION

Differentiating muscle cells fuse to form multinucleated myotubes, thereby withdrawing permanently from the cell cycle. In mice, specification as well as differentiation of skeletal muscle cells is dependent on four muscle-specific basic helix-loop-helix transcription factors, the *MyoD* family of muscle-determination genes. Targeted disruption of each family member *(MyoD, Myf-5, myogenin,* and *MRF4)* in mice has demonstrated the importance of these factors for vertebrate myogenesis. Inactivation of *Myf-5* results in defects in early myotome formation with presumptive muscle precursors adopting nonmuscle cell fates *(7,88,89).* However, this early myotomal defect is compensated by *MyoD,* leading to apparently normal muscle at birth. Targeted disruption of *MyoD* alone also does not dramatically affect muscle specification or differentiation, as these mice have grossly normal muscle *(76).* The functional overlap between *Myf-5* and *MyoD* was demonstrated by generating mice with disruption of both genes, resulting in a complete absence of proliferating myoblasts as well as differentiated muscle fibers *(77).* In contrast, mice with disruption of the *myogenin* gene contain normal numbers of proliferating myoblasts, but these cells fail to differentiate, indicating that myogenin has a unique role in the transition from determined myoblasts to a fully differentiated

myotubes *(41,61)*. Lastly, inactivation of the *MRF4* gene results in a very mild muscle phenotype, consistent with the notion that *MRF4* may have functions that are redundant with the other *MyoD* family members *(6,68,104)*. These experiments have led to a simple model for vertebrate-skeletal myogenesis that involves Myf-5 and MyoD functioning early to establish or determine myoblast-cell fate, and myogenin mediating the terminal differentiation of myoblasts.

Vertebrate-skeletal muscle is derived from cells in the prechordal and somitic mesoderm that give rise to committed myogenic cells of the somite, which become the skeletal muscle of the head, trunk, and limbs. The different myofiber and myogenic constituents are thought to be formed from different lineages of myogenic cells *(58)*. In the mouse, primary myofibers develop first at 8.5 d of gestation, followed by secondary myofibers at day 14. Recent experiments have suggested that MyoD and Myf-5 do have distinct roles for determining these myogenic lineages during development *(45)*. Although *MyoD*$^{-/-}$ embryos display normal development of paraspinal and intercostal muscles in the body proper, muscle development in limb buds and brachial arches is delayed by about 2.5 d. By contrast, *Myf-5*$^{-/-}$ embryos display normal muscle development in limb buds and brachial arches, and markedly delayed development of paraspinal and intercostal muscles. Taken together, these observations strongly support the hypothesis that Myf-5 and MyoD play unique roles in the development of epaxial and hypaxial muscle, respectively. In addition, satellite cells, the muscle stem cells of adult muscle, arise around day 17 as a unique myogenic lineage (for review, *see* ref. *5)*. Satellite cells are mitotically quiescent but are induced to enter the cell cycle in response to stress induced by exercise or injury. The activated satellite cells undergo multiple rounds of division before fusing with existing myofibers resulting in repair and/or hypertrophy. Recently, MyoD has been shown to play a novel role in satellite-cell function *(56)*. *MyoD*$^{-/-}$ mice interbred with the *mdx* mouse (a model for Duchenne and Becker muscular dystrophy) exhibit increased penetrance of the *mdx* phenotype characterized by reduced muscle hypertrophy and increased myopathy. This condition is thought to arise owing to a defect in muscle regeneration. Consistent with this hypothesis, the single mutant *MyoD*$^{-/-}$ knockout mice show defects in skeletal-muscle regeneration following injury *(56)*.

5. CONTROL OF MYOD ACTIVITY BY pRB

Tissue-culture experiments have been useful in elucidating the sequence of events that result in terminal cell-cycle withdrawal and differentiation of muscle cells. This process is controlled by regulatory interactions involving MyoD family members and various cell-cycle-related proteins (reviewed in refs. *48,66)*. Myf-5 and MyoD are present in proliferating myoblasts and initiate a cascade of events, including activation of myogenin expression when myoblasts are deprived of mitogens, resulting in terminal differentiation. Furthermore, MyoD transactivation of muscle-specific genes to high levels requires pRB *(34,63)* or high levels of the pRB related protein p107 *(79)*. Loss of pRB function in muscle cells has two prominent phenotypes: (1) the level of activation of terminal differentiation markers is drastically reduced, and (2) new DNA synthesis can occur in the nuclei of the rare differentiated myotubes. During muscle differentiation, pRB expression increases *(15,26,54)* and assumes a hypophosphorylated and activated state *(34,91)*. These studies show that pRB plays a critical role in myogenesis.

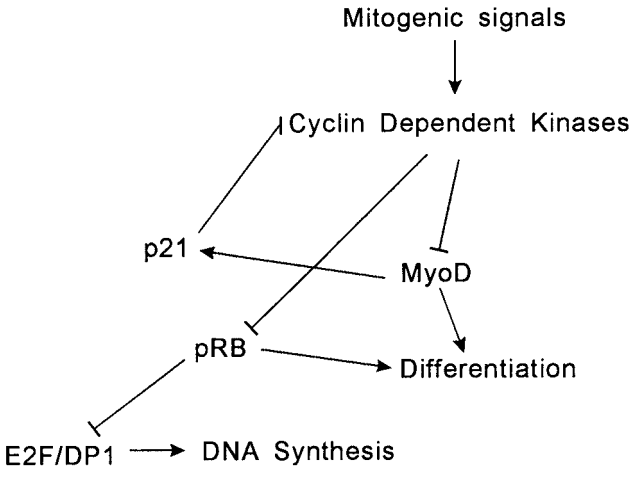


Fig. 2. Model for muscle-cell differentiation.

However, the precise mechanism by which pRB participates in myogenesis remains unclear. Direct protein-protein interaction between pRB and MyoD has been proposed to account for the pRB requirement *(34).* However, it remains possible that the affects of pRB on muscle differentiation is an indirect affect via one or more cell-cycle changes mediated by pRB. In addition, MyoD is thought to induce cell-cycle arrest during differentiation, at least in part, by inducing expression of the cyclin-kinase inhibitor p21, which would in turn inhibit the G1-phase cyclins and allow pRB to become activated *(35,37).* A current model for muscle-cell differentiation is shown in Fig. 2.

6. MYOD AND ONCOGENESIS

In addition to promoting tumor formation, anchorage-independent growth, and cellular immortalization, expression of transforming oncogenes inhibits cellular differentiation in several different cell lineages *(4,14,21,29,30,43,49,70).* In muscle cells, for example, expression of oncogenic tyrosine kinases (v-src and v-fps), growth-factor receptors (v-erbB), nuclear oncogenes (v-myc, c-myc, v-erbA, and E1A), and the activated form of signal-transducing G proteins (H-ras and N-ras) can inhibit terminal differentiation to varying extents *(27,29,33,43,67,80,95).* Furthermore, several hypotheses have been proposed to explain how the MyoD family of proteins are kept in check during proliferation: (1) inhibition of the MyoD family members by interaction with the Id family of negative HLH factors *(3);* (2) inhibitory phosphorylation of the MyoD family members by protein kinase C *(39,50);* and (3) inhibition by cyclin-D-dependent kinases *(73,84).* However, none of these interactions have been shown to be causative in the generation of the abnormal proliferation or lack of differentiation of skeletal-muscle tumors.

7. MYOD AND SKELETAL-MUSCLE TUMORS

Rhabdomyosarcomas are skeletal-muscle tumors and are one of the more common solid tumors of childhood, representing 4–8% of all malignancies in humans under 15 years of age. Tumors arise de novo from skeletal muscle. Sarcomas have traditionally been classified as rhabdomyosarcomas based on morphology and expression of muscle-

specific structural genes, such as myosin heavy chain or desmin. Expression of *MyoD* has been shown to be the most sensitive marker for classifying sarcomas as rhabdomyosarcomas *(20,81).* This is paradoxical, because MyoD has been shown to induce muscle differentiation in a wide variety of primary cells and transformed cell lines *(96),* yet rhabdomyosarcomas have a low propensity to differentiate into myotubes. Thus, even though MyoD is expressed in rhabdomyosarcomas cells, it would appear to be nonfunctional in inducing differentiation in these cells.

Rhabdomyosarcomas are grouped by histologic and cytogenetic criteria as either embryonal or alveolar rhabdomyosarcomas: a balanced translocation between chromosomes 2 and 13, t(2:13)(q35;q14), is associated with alveolar rhabdomyosarcomas *(2).* The *PAX3* gene has been shown to be fused to a forkhead gene-family member *(FKHR)* in the t(2:13) translocation *(2,82).* More recently, the *PAX7* gene, located at 1p36, has been shown to be translocated to the *FKHR* gene as a 1:13 translocation (p36;q14) in some cases of alveolar rhabdomyosarcoma *(17).* Loss of heterozygosity on the short arm of chromosome 11 encompassing 11p15 is associated not only with embryonal rhabdomyosarcomas *(81)* but also with several other solid tumors *(62),* suggesting the location of one or more tumor-suppressor genes for multiple tumor types in this region. In addition, gene amplification has been observed in both embryonal and alveolar rhabdomyosarcomas. Comparative genomic hybridization showed that in primary alveolar rhabdomyosarcomas the most frequent amplicons are localized to 2p24 and 12q13-14, with both amplifications occurring in 4 out of 10 tumors *(94).* The 2p24 amplicon had previously been shown to involve the *MYCN* gene *(19),* whereas the genes involved in the 12q13-14 amplicon have not yet been fully defined. Two distinct chromosome 12q13-14 amplicons have been described in other types of sarcomas *(87)* as well as in gliomas *(74).* Mapping of these two amplicons implicates *MDM2* or *CDK4* and *SAS* as likely targets of the amplification events *(74).* The frequency of these two different amplicons in primary rhabdomyosarcomas is currently unknown.

8. INHIBITORS OF MUSCLE DIFFERENTIATION

8.1. Amplification of MDM2

One obvious phenotype of tumor cells is a lack of terminal differentiation. We have conducted a series of somatic-cell genetic experiments designed to identify genetic loci present in rhabdomyosarcoma cells lines that are capable of inhibiting muscle differentiation *(28,85,90).* Initially, we showed that rhabdomyosarcomas could be classified as either dominant or recessive with respect to MyoD activity and terminal differentiation *(90).* Subsequent analysis of two different dominant types of tumors indicated that the loss of differentiation could be mapped to individual loci. First, microcell-mediated chromosome transfer of a derivative chromosome 14 from the rhabdomyosarcoma cell line Rh18 into the differentiation competent myoblast cell line C2C12 inhibits muscle differentiation and the ability of MyoD to transactivate reporter constructs. The derivative chromosome 14 contains a region of amplified DNA originating from chromosome 12q13-14, and contains several genes often amplified in sarcomas *(28).* Testing the amplified genes for the ability to inhibit muscle-specific gene expression indicates that forced expression of *MDM2* inhibits MyoD function, and consequently inhibits muscle differentiation. Thus, amplification and overexpression of *MDM2* in rhabdomyosarcomas inhibits MyoD function, resulting in dominant inhibition of muscle differentiation *(28).*

The oncogenic properties of MDM2 have been postulated to result from direct interaction with several cell-cycle regulatory proteins. MDM2 interacts directly with p53 *(64),* and blocks p53-mediated transactivation by inhibiting the activation domain of p53 *(12,36,59,65,98,103).* In addition, MDM2 interacts with the activation domain of E2F1, resulting in stimulation of E2F1/DP1 transcriptional activity *(55).* Furthermore, MDM2 has been shown to interact directly with pRB, resulting in stimulation of E2F/DP1 transcriptional activity and inhibition of pRB growth-regulatory function *(99).* Taken together, these results suggest that MDM2 not only relieves the proliferative block mediated by either p53 or pRB, but also promotes proliferation by stimulating the S phase-inducing transcriptional activity of E2F/DP1. Thus, the most obvious role for amplification of *MDM2* in tumorigenesis would be to inactivate p53 and/or pRB. In addition, we have shown that amplification of *MDM2* inhibits MyoD activity and consequently inhibits normal muscle differentiation *(28).* These studies have identified a previously unknown activity for the MDM2 protein, and broadens the role of MDM2 in cell-growth control to include inhibition of differentiation.

Because MDM2 is expressed in proliferating myoblasts, and MDM2 can inhibit muscle differentiation *(28),* one possibility is that MDM2 inhibits differentiation of myoblasts during cellular proliferation. Furthermore, activation of muscle-specific genes to high levels requires members of the pRB family *(34,63,79).* During muscle differentiation, pRB expression increases *(15,26,54)* and assumes a hypophosphorylated, activated state *(34,91).* Because MDM2 interacts with pRB and inhibits the growth-regulatory functions of pRB *(99),* it is possible that pRB in turn regulates the growth-stimulatory effects of MDM2. Consistent with this possibility, we find that forced expression of pRB can restore MyoD activity in cells with amplified *MDM2* (unpublished observations). Because pRB and MDM2 interact, these results suggest that an additional role for pRB during muscle-cell differentiation is to bind and inactivate MDM2.

8.2. Duplication of ATR

In a second screen for inhibitors of differentiation, we found that microcell-mediated chromosome transfer of an isochromosome 3q [i(3q)] from a different rhabdomyosarcoma cell line into the differentiation-competent myoblast cell line C2C12 also inhibits differentiation. In addition to inhibition of differentiation, the i(3q) causes abnormal centrosome amplification, resulting in aneuploidy, and abolishes G1 arrest following DNA damage *(85).* We have extended these observations by showing that forced expression of *ATR* (located at 3q24) results in a phenocopy of the i(3q) containing hybrids. These findings may have implications for nonmuscle tumors as well. Comparative genomic hybridization was used to demonstrate that 3q is a hotspot for increased DNA copy number in several different types of cancers. In head and neck squamous-cell carcinoma, the most frequently observed increase in DNA copy number was from chromosome 3q, occurring in 10 of 13 primary tumors *(86).* Karyotypic analysis of these cells showed the presence of an i(3q) in 30–40% of tumors *(11,44,72).* Similarly, the most frequent increase in DNA copy number in primary small-cell lung carcinomas was also on 3q, occurring in 10 out of 13 cases *(75).* Furthermore, the formation of an i(3q) has been observed in small-cell lung carcinomas *(71).* Prior to our study, the most compelling argument for an involvement of 3q in tumorigenesis came from analysis of

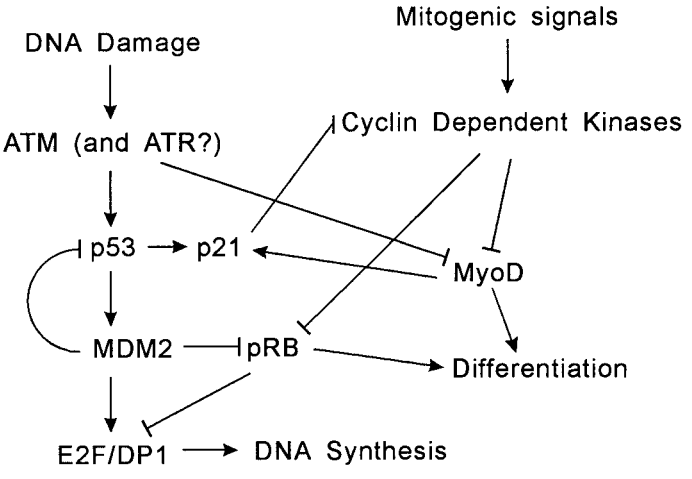


Fig. 3. Model integrating G1 checkpoint and muscle-cell differentiation.

cervical carcinomas *(42).* Gain of 3q is the most consistent chromosomal aberration in cervical carcinomas and was present in 9 out of 10 tumors. Furthermore, this alteration occurs during the progression from severe dysplasia to invasive carcinoma *(42).* Interestingly, the gain of 3q correlated with the conversion of tetraploid dysplastic cells to aneuploid carcinoma cells. Although it remains possible that different genetic alterations of 3q occur in different tumors, our data indicate that the i(3q) present in Rh30 rhabdomyosarcoma cells induces aneuploidy. Therefore, the aneuploidy observed in cervical carcinoma cells may be a direct result of increased copy number on 3q resulting in *ATR* overexpression. Taken together, these studies implicate alterations in the *ATR* locus as causing loss of differentiation and cell-cycle abnormalities in several different types of tumors.

9. SUMMARY: CELL-CYCLE CHECKPOINTS AND DIFFERENTIATION

Because both cell-cycle checkpoints and muscle differentiation use the same cyclin-kinase inhibitor, p21, to arrest the cell cycle, and because forced expression of p21 in muscle cells leads to premature differentiation *(37),* a mechanism to protect the myoblast population from premature differentiation induced by DNA damage must exist. We propose that the inhibitory interactions between MDM2 and MyoD and between ATR and MyoD fulfill this role, so that if a myoblast sustains DNA damage, premature differentiation does not occur. A model to integrate these two processes is shown in Fig. 3.

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13

Ultraviolet Light-Induced and Spontaneous Recombination in Eukaryotes

Roles of DNA Damage and DNA Repair Proteins

Colin A. Bill and Jac A. Nickoloff

1. INTRODUCTION

The major biological effects of ultraviolet (UV) radiation are caused by DNA damage (for reviews see Vol. 1, Chapters 15, 17, and 18; Vol. 2, Chapter 15, and refs. *120,181*). UV damage can result in genetic mutations that may promote tumorigenesis (*205*). Indeed, solar UV is the primary cause of skin cancer, the most prevalent form of cancer (*21,94,189*). There is concern about loss of the stratospheric ozone layer, which may result in additional exposure of the earth's surface to UV and consequently an even greater incidence of skin cancer. Therefore, it is important to determine the genetic effects of UV damage. One such effect is recombination, which can result in genetic rearrangements that may contribute to carcinogenesis.

There are three types of UV radiation: UVA (320–400 nm), UVB (280–320 nm), and UVC (200–280 nm). Although UVC is the most damaging to cells, only UVA and UVB penetrate the atmosphere, with most of the deleterious effects attributable to UVB (*181*). UVB and UVC produce more than a dozen photoproducts in DNA. Among these, cyclobutane pyrimidine dimers (CPDs) and pyrimidine-pyrimidone (6–4) photoproducts [(6–4)PDs] are the most common, comprising ~75% and ~25% of the total, respectively (*65,119*). UV radiation acts as both initiator and promoter of carcinogenesis, and it is likely that these properties are owing to CPD and (6-4)PD damage (*95,205*). Because the earth is constantly exposed to UV, it is not surprising that cells have evolved several DNA repair mechanisms that protect genetic information from the harmful effects of solar UV radiation (*29,63*).

1.1. Repair of UV-Induced DNA Damage

Most UV lesions in DNA are repaired by nucleotide-excision repair (NER). In eukaryotes this pathway involves incision on either side of a lesion followed by excision of 27–29 nucleotide oligomers. The remaining undamaged DNA strand is used as a template for DNA synthesis to restore the double-stranded DNA (Vol. 1, Chapter 15). In humans, defects in NER cause UV hypersensitivity and have been associated with three

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hereditary diseases, xeroderma pigmentosum (XP), Cockayne's syndrome, and trichothiodystrophy (Vol. 2, Chapter 18). Repair of UV-induced DNA lesions is heterogeneous across the genome, with the actively transcribed strands preferentially repaired over nontranscribed strands of active genes or nontranscribed DNA (*17,116,188*). Such transcription-coupled repair (TCR) is conserved from bacteria to mammals (Vol. 1, Chapter 9 and Vol. 2, Chapters 10 and 18). DNA lesions in nontranscribed sequences or in inactive chromatin are repaired by a general NER pathway termed "global genome repair."

In addition to NER, UV lesions are repaired by several alternative pathways (*203*). Photolyase reverses UV damage by a visible light-dependent reaction called photoreactivation (see Vol. 2, Chapter 2 and ref. *152*). Photolyases specific for CPDs and (6-4)PDs have been identified (*179*). There are also UV damage-specific glycosylases, which produce a nick at one of the glycosyl bonds in a dimer, generating an abasic site that is a substrate for base-excision repair (BER) (*63*). Another mechanism involves a UV-damage endonuclease (UVDE), which introduces an incision 5′ to CPDs and (6-4)PDs and initiates a excision-repair process (*19,201*). Most UV damage is repaired rapidly, although some lesions may persist through several cell divisions before being repaired, producing mutations, or stimulating recombination.

1.2. Cellular Effects of UV-Induced DNA Damage

Cells exposed to UV may undergo cell-cycle arrest, usually at S phase or the G1/S boundary, and then repair or tolerate the damage before resuming the cell cycle, or they may die, often by apoptosis (*153*). Although UV lesions can inhibit DNA synthesis, lesions may persist because of bypass mechanisms (*30,175*). It is well-established that bulky DNA lesions such as CPDs and (6-4)PDs block transcription elongation in vivo and in vitro (*48,187*). Partial and often nonfunctional RNA transcripts are formed from genes containing one or more lesions in transcribed strands. Eukaryotes also respond to UV damage by inducing transcription of a large set of genes. This gene induction facilitates cell-cycle arrest, DNA repair, and adaptation to the insult (*5,44–46,80*). A major consequence of UV irradiation is enhanced recombination. Recombination is a fundamental process in normal metabolism and repair of DNA damage (reviewed in refs. *131,176*). Recombinational repair can restore genetic information but also can produce mutations and chromosome rearrangements that may have deleterious consequences. For example, recombination may contribute to carcinogenesis by causing loss of heterozygosity that inactivates tumor-suppressor genes (e.g., *p53*), or by activating oncogenes by gene duplication, amplification, inversion, deletion, or translocation.

Recombination can occur between homologous or nonhomologous sequences. Homologous recombination involves interactions between DNA sequences sharing significant lengths of homology (>200 bp). Typical homologous recombination substrates are diagrammed in Fig. 1A. Interacting regions may be at allelic positions on homologs (in diploid cells), termed "allelic recombination." Ectopic recombination includes a variety of interactions between nonallelic repeats, such as repeats present on a single chromosome (direct or inverted orientations), at nonallelic positions on homologs, or on nonhomologous chromosomes. Recombination can be conservative, including reciprocal exchanges (crossing-over) and nonreciprocal exchange (gene conversion), or it can be nonconservative, such as half-crossovers or intrachromosomal deletions between direct repeats via single-strand annealing (SSA). In SSA, DNA ends are processed to

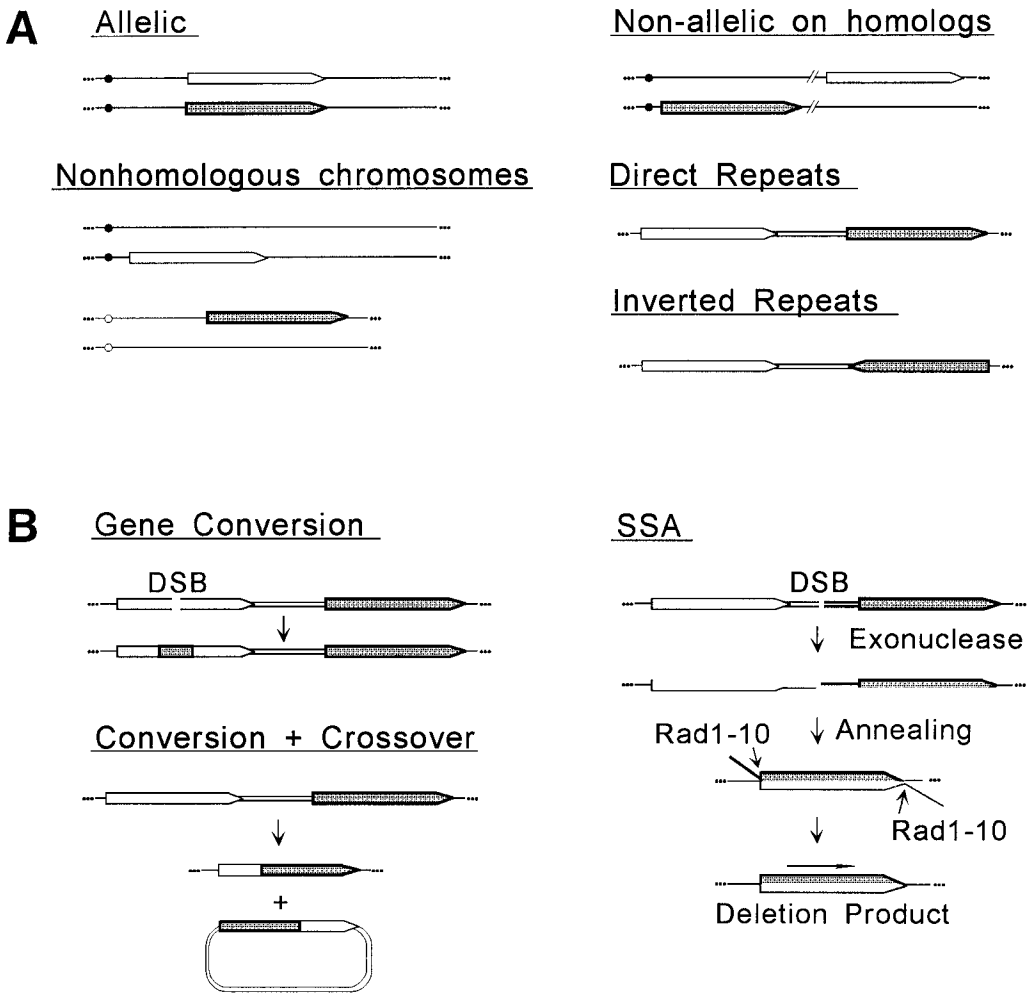


Fig. 1. (A) Types of recombination substrates and events. Repeated regions are shown by boxes, with open and shaded boxes indicating different alleles. Allelic interactions occur between genes present at identical positions in homologous chromosomes. All other interactions are ectopic, including interactions between nonallelic repeats on homologs, repeats on nonhomologous chromosomes, and linked repeats in direct or inverted orientation. **(B)** Types of recombination events. Gene conversion can occur between any repeated regions, shown here for direct repeats. Gene conversion is a nonreciprocal transfer of information from a donor locus (shaded) to a recipient locus (open); typically a damaged allele is the recipient. Gene conversion conserves the gross structure of the recombination substrate. Conversions are often associated with crossovers, which in direct repeats leads to a deletion product plus an excised, circular product that is normally lost. For nonallelic interchromosomal interactions, crossovers result in translocations. Depending on the arrangement of the interacting alleles with respect to centromeres, translocations may be balanced or unbalanced, with unbalanced translocations producing dicentric and acentric fragments (not shown). For inverted repeats, conversion associated with a crossover leads to inversion of DNA between repeats (not shown). SSA occurs between direct repeats and may be initiated by a DSB that is processed by a single-strand exonuclease to expose single-stranded complementary regions. Annealing produces 3′ tails at duplex DNA junctions that are processed by Rad1p/10p endonuclease to produce a deletion product similar to a crossover product. However, unlike crossing over, SSA is nonconservative because no circular product is formed.

expose single-stranded regions that can anneal to form an apparent crossover product with deletion of the DNA between repeats. SSA is common in direct-repeat recombination in yeast (*133*). In mammalian cells, SSA is the predominant mode of extrachromosomal recombination (*42,101,102*), but gene conversion is predominant for chromosomal events (*18,103,130,173*). Sister chromatid recombination (including sister chromatid exchange [SCE] and gene conversion) can be detected at the molecular level only for interactions between linked repeats. In mammalian cells, SCE can be detected in whole chromosomes by microscopic examination. Each type of recombination substrate provides a limited view of the possible recombination events. Recombination substrates are often designed with selectable markers to allow detection of rare events, but selection strongly restricts the types of events detected. Nonhomologous recombination includes nonhomologous end-joining (NHEJ), a mechanism for repairing double-strand breaks (DSBs), and the integration of exogenous DNA into a nonhomologous chromosomal locus (described herein as illegitimate recombination). In this review we focus on the mechanisms and genetic control of UV-induced recombination in eukaryotic cells with emphasis on the yeast *Saccharomyces cerevisiae* and mammalian cells. We also discuss the effects of mutations in UV-repair genes on spontaneous and UV-induced recombination because these provide important insights into recombination mechanisms. For a discussion of recombinational repair in prokaryotes, see the recent review by Cox (*39*).

2. UV-INDUCED RECOMBINATION IN *SACCHAROMYCES CEREVISIAE*

Resistance to the cytotoxic effects of UV in *S. cerevisiae* reflects three types of mechanisms, including NER mediated by genes in the *RAD3* epistasis group (Vol. 1, Chapter 15), damage tolerance mechanisms (so-called error-prone or mutagenic-repair pathways such as translesion synthesis) mediated by genes in the *RAD6* epistasis group (*63*), and recombinational repair of DSBs mediated by genes in the *RAD52* epistasis group (see Vol. 1, Chapter 16 and ref. *133*). DSBs are highly recombinogenic lesions. It is thought that UV or the repair of UV damage does not directly produce double-stranded damage such as DSBs (except perhaps at very high doses), yet UV is highly recombinogenic in yeast and there is considerable indirect evidence suggesting that a significant fraction of UV-induced recombination in yeast involves a DSB intermediate. Yeast is either homozygous or heterozygous at the mating-type locus, *MAT,* and *MAT* genotype strongly influences recombination and DSB repair. *MAT* status influences both UV survival (in UV-sensitive mutants) and UV-induced recombination; these *MAT*-specific topics are discussed in Chapter 5.

2.1. Potential Roles for DSBs and Replication in UV-Induced Recombination

There are several reasonable models for how UV damage might stimulate recombination. (1) UV damage might stimulate recombination by altering DNA structure, perhaps by inducing bending (*81,174*) because bending influences recombination (*147,162*). However, the degree of bending at UV dimers has been questioned (*192,202*) and there is no direct evidence that bending at UV damage stimulates recombination. (2) Recombination might be enhanced as a consequence of UV repair. The principal mechanism of UV repair is NER, which exposes short single-stranded regions that might promote pairing/strand exchange with regions of homology elsewhere in the

genome. However, this view is inconsistent with many studies that indicate that UV-induced recombination usually increases when NER is disabled (Subheadings 3.1. and 5.1.). (3) UV damage might stimulate recombination indirectly, i.e., at undamaged loci, perhaps as a result of gene induction (Subheadings 2.2.). (4) An early model to account for UV-induced recombination in *Escherichia coli* suggested that UV-induced postreplication gaps serve as regions for strand exchange (*149*). (5) Increasing evidence favors the idea that single-strand damage such as UV dimers can be converted to recombinogenic DSBs during replication.

UV lesions may be converted to DSBs when replication forks encounter single-strand breaks (SSBs) or gaps resulting from incomplete processing of UV damage by NER. However, this would predict that recombination would depend on NER and this is not the case (Subheadings 3.1. and 5.1.). Alternatively, DSBs could arise when a replication fork encounters a post-replication gap. Following UV irradiation of bacteria, daughter strands gaps appear at intervals approximating the locations of lesions on the template strands, suggesting that these gaps arise during replication bypass of UV lesions (reviewed in ref. *39*). An arrested replication fork can be processed into a double-stranded end by fork reversal or direct breakage (reviewed in refs. *39,75,93,110,148*). Bacterial studies have shown that replication forks that arrest as a consequence of metabolic processes or DNA damage can restart by a recombinational mechanism, a process termed recombination-dependent DNA replication (*6,93*). DSBs at arrested replication forks have been observed in *E. coli,* in a mechanism dependent on the Holliday junction resolving and branch-migration enzyme complex RuvABC (*118,157*). DSBs have been directly observed following exposure to UV in both yeast and mammalian cells (*61,194*), and there is evidence that a significant fraction of yeast-cell killing by UV results from such DSBs (*91*).

Members of the *RAD52* epistasis group, such as *RAD50, RAD51,* and *RAD54* are important for recombinational repair of DSBs, and *RAD52* is essential for nearly all types of DSB-induced recombination (*133*). A number of studies have shown that UV-induced recombination is dependent on *RAD52* and other *RAD52* group members, consistent with the idea that UV-induced recombination proceeds through a DSB intermediate. For example, UV-induced sister chromatid recombination is eliminated in *rad52* mutants, and reduced by half in *rad50* (end-processing) mutants (*87*). The enhancement of integrative transformation by UV treatment of plasmid DNA is completely abolished in *rad52* mutants (*150*). The UV repair protein Rad3p is a helicase that is essential for viability and NER. Some mutant *rad3* alleles, termed *rem,* display a hyper-recombination phenotype and are synthetically lethal with *rad52 (121).* Song et al. *(163)* argued that the hyper-recombination seen in *rem* strains reflects enhanced conversion of spontaneous damage to DSBs, thus accounting for the *RAD52*-dependence of *rem*-enhanced recombination.

More direct evidence that UV-induced recombination proceeds through a DSB intermediate in yeast comes from studies of chromosomal recombination between partial, nontandem direct repeats *(64).* To stimulate recombination, I-*Sce*I or gpII recognition sequences were located between the repeated genes to allow targeted DSBs and SSBs, respectively, or by exposing cells to γ or UV radiation. Because the recombination substrates were themselves temperature-sensitive mutants of essential genes (*cdc28* and *tub1*), recombination could be studied in arrested cells at the restrictive temperature, or in

dividing cells at the permissive temperature. As expected, DSBs induced by I-*Sce*I nuclease or γ-rays enhanced recombination, both in dividing and nondividing cells. In contrast, SSBs created by gpII showed modestly enhanced recombination in dividing cells, but no enhancement in arrested cells, suggesting that recombination required conversion of SSBs to DSBs during replication. Interestingly, moderate UV doses induced recombination only in dividing cells, but high UV doses enhanced recombination in both dividing and nondividing cells. These results are consistent with the idea that single-strand damage at moderate UV doses is converted to recombinogenic DSBs during replication, whereas at high doses, DSBs may be produced directly by NER processing of closely opposed lesions, obviating the need for lesion conversion during replication. In *rad1* mutants, SCE is replication-dependent, providing additional evidence that replication converts UV damage into recombinogenic lesions *(138),* although the same SCE assay in *RAD1* cells showed a significant component of replication-independent SCE *(86).* The role of Rad1p in UV-induced recombination is considered further in subheading 3.1.

2.2. *Indirect Stimulation of Recombination*

Recombination between chromosomes of unirradiated cells is induced following mating with UV-irradiated cells *(51).* Because the indirect induction of recombination persists for several cell generations *(50),* it seems unlikely that it results from persistent DSBs, although persistence of the primary lesions (which may later be converted to DSBs) remains a possibility. Lesions may persist owing to tolerance mechanisms such as translesion synthesis and lesion bypass (reviewed in ref. *63).* UV lesions may stimulate recombination in undamaged DNA by a triparental mechanism, as with DSBs *(146).*

Alternatively, indirect stimulation of recombination by UV might reflect induction of genes that enhance recombination, an idea supported by several lines of evidence. For example, many genes are induced by UV and other DNA-damaging agents, including several with key roles in recombination such as *RAD51, RAD52,* and *RAD54 (1,38).* The induction of *RAD51* and related genes by UV is conserved through evolution, as UV induces human *RAD51* and *RAD51L1* (also known as *REC2* and *RAD51B*) *(139).* Overexpression of yeast *RAD52* in human cells increases extrachromosomal recombination *(84),* and overexpression of human *hRAD51* increases recombination in human cells *(200).* Also, the UV-sensitivity of yeast *rad51* mutants can be suppressed by overexpression of *RAD54 (36)* (Subheading 3.5.). Although the immediate stimulatory effects of UV on recombination might be at least partially owing to induction of *RAD51, RAD52,* and *RAD54* and/or other genes (reviewed in ref. *62),* the observed indirect stimulation persists much longer than gene induction. Indirect stimulation of recombination may reflect the combined effects of lesion persistence, lesion transfer, triparental recombination, and gene induction.

3. RECOMBINATION IN YEAST MUTANTS WITH DEFECTS IN DNA REPAIR

3.1 *Recombination in RAD3 (NER) Epistasis Group Mutants*

The *RAD3* epistasis group is comprised of a large number of genes that encode proteins involved in the repair of UV damage; those with known effects on recombination are listed in Table 1. Of these, *RAD1* and *RAD10* have been studied most extensively.

Table 1
Partial Listing of *S. cerevisiae* Genes Involved in UV Damage Repair, Tolerance, Checkpoints, and Recombination

Gene	Biochemical activity and function	Reference
***RAD3* epistasis group:**		
RAD1	Complexes with Rad10p, cleaves 3′ single-stranded tails at single-strand/duplex DNA junctions	(Vol. 1, Chapter 15)
RAD2	Endonuclease cleaves 5′ single-stranded tails at single-strand/duplex DNA junctions	(Vol. 1, Chapter 15)
RAD3	5′→3′ helicase, DNA-dependent ATPase, unwinds DNA at lesion	(Vol. 1, Chapter 15)
RAD4	Complexes with Rad23p, UV damage recognition	*(73)*
RAD7	UV-inducible, required for repair of nontranscribed DNA	(Vol. 1, Chapter 15)
RAD10	Complexes with Rad1p, cleaves 3′ single-stranded tails at single-strand/duplex DNA junctions	(Vol. 1, Chapter 15)
RAD14	UV damage recognition	(Vol. 1, Chapter 15)
RAD23	UV-inducible, complexes with Rad4p, UV damage recognition	(Vol. 1, Chapter 15 and ref. 73)
DUN1	Protein kinase, controls transcriptional response to DNA damage; partial checkpoint defect	*(8,54)*
SSL1	Essential gene involved in transcription and NER, component of TFIIH	(Vol. 1, Chapter 15)
***RAD6* epistasis group:**		
RAD5	DNA-dependent helicase, zinc finger motif	(Vol. 1, Chapter 15 and ref. *85)*
RAD6	UV-inducible, ubiquitin-conjugating enzyme	(Vol. 1, Chapter 15)
RAD18	UV-inducible, binds ssDNA	(Vol. 1, Chapter 15)
Checkpoint genes:		
RAD9	Controls G1/S, G2/M checkpoints; partial control of S checkpoint	(Vol. 1, Chapter 17)
RAD17	Controls G1/S, G2/M, and meiosis I checkpoints; partial control of S checkpoint	(Vol. 1, Chapter 15)
RAD24	Controls G1/S, G2/M, and meiosis I checkpoints; partial control of S checkpoint	(Vol. 1, Chapter 17)
***RAD52* epistasis group and other genes:**		
RAD51	UV-inducible, strand exchange, and homologous pairing activities; mutant mildly UV-sensitive	(Vol. 1, Chapter 16 and ref. *133)*
RAD52	UV-inducible, DNA end-binding activity, activates Rad51p	(Vol. 1, Chapter 16 and ref. *133)*
RAD54	UV-inducible, helicase (?), activates Rad51p	(Vol. 1, Chapter 16 and ref. *133)*
EXO1	UV-inducible, structure-specific endonuclease, acts in NER-independent UV repair	*(143)*
PSO4	Repair and recombination defects, role in mRNA splicing *(PRP19),* also in *RAD6* epistasis group	*(68)*

For a complete listing of UV repair and checkpoint genes, see Vol. 1, Chapters 15 and 17.

The Rad1p/10p complex is a structure-specific endonuclease that cleaves 3′ single-stranded tails at the junctions of single-stranded and duplex DNA. As such, Rad1p/10p is important for SSA and DSB-induced gene conversion when the broken ends are not fully homologous to donor sequences (Fig. 1B) *(83),* although there is a minor Rad1p-independent pathway for removing nonhomologous single-stranded tails *(37).*

Mutants defective in NER allow one to test the hypothesis that recombination is enhanced by strand breaks and/or single-stranded regions created during NER. In this view, NER-defective mutants would be expected to have lower levels of UV-induced recombination than NER-proficient cells. Although various NER mutants have different effects on UV-induced recombination depending on the type of recombination substrate and cell-cycle phase, most NER mutants display increased levels of UV-induced recombination. Thus, recombination is enhanced by DNA damage, not by repair.

Kadyk and Hartwell *(86)* showed that NER-defective *rad1* mutants display higher frequencies of UV-induced sister chromatid recombination than wild-type. The sister chromatid recombination assay measured both gene conversion and reciprocal exchange. These events in *rad1* cells were dependent on replication: there was no stimulation in cells irradiated in G2 until they passed through the next round of replication. These results are consistent with the replication-dependent conversion of UV lesions to recombinogenic (DSB?) lesions. In contrast, allelic recombination showed a dose-dependent increase in Rad$^+$ cells, but no induction was seen at any dose in *rad1* mutants. There appear to be at least two distinct mechanisms of UV-induced sister chromatid recombination because *rad1* mutants gave rise exclusively to gene conversions, whereas *RAD1* cells gave rise to 75% gene conversion and 25% reciprocal exchange *(86).*

Integrative transformation of UV-damaged (nonreplicative) plasmid DNA showed a clear dose-dependent increase in Rad$^+$ cells. A much sharper increase at a low dose was seen in *rad1* and *rad3* mutants, but there was no further increase at higher doses *(150),* suggesting a saturated process. In Rad$^+$ cells a significant fraction of transformants had multiple copies of the integrated plasmid and the number of copies increased with UV dose. Multiple copies likely arise via SSA that can produce concatemers that subsequently integrate into the target chromosome. Because Rad1p/10p endonuclease is required to process SSA intermediates *(83),* it is not surprising that *rad1* mutants yielded more gene conversions and fewer transformants with multiple integrated copies; in this system gene conversion does not require Rad1p/10p. In *rad3* and *rad4* mutants, conversions were as rare as in Rad$^+$ *(150),* indicating that the shift from integration toward gene conversion in *rad1* is independent of the *rad1* NER defect.

A comprehensive study of NER mutants revealed a variety of effects on spontaneous recombination, including Ty and non-Ty direct repeat recombination, and ectopic Ty and non-Ty gene conversion *(99).* In a *rad1* mutant, direct-repeat recombination decreased slightly at Ty and decreased by ~threefold at the non-Ty substrate. In contrast, conversion increased by ~twofold at Ty and non-Ty loci. Interestingly, *rad10* had similar effects on both types of direct-repeat recombination, but no effect on conversion, suggesting that Rad1p and/or Rad10p have roles that are independent of their endonucleolytic roles in the Rad1p/10p complex. Because *rad1* had stronger effects, it was not surprising that the recombination phenotype of the *rad1 rad10* double mutant was essentially the same as *rad1.* Mutations in *rad2, rad4, rad7, rad14,* and *rad23* had little or no effect on any of these recombination endpoints. The decrease in direct-repeat

recombination in the *rad1* and *rad10* single mutants, and the double mutant, can be understood in terms of the important role that Rad1p/10p endonuclease plays in SSA (Fig. 1B). It was suggested that the increase in conversion in *rad1* reflects channeling of DNA damage from direct-repeat recombination toward conversion, although this does not adequately explain the lack of effect in other NER-defective mutants (*rad2,4,7,10,14,* and *23*). Together these data suggest a role for *rad1* in gene conversion that is independent of its role in NER, perhaps through interactions with the mismatch repair (MMR) system (Subheadings 3.2. and 3.4.).

Deletions in direct-repeat recombination substrates are not solely a consequence of SSA. A study of spontaneous deletions in short direct repeats in wild-type cells and *rad1* mutants revealed that *rad1* reduced deletions by 10-fold with 415 bp repeats, indicative of an SSA defect, but only fivefold with 223 bp repeats, and only threefold with 103 bp repeats (A. Bailis, personal communication). Because Rad1p/10p is required to remove nonhomologous termini >60 bp in length during SSA *(59),* it appears that a significant fraction of these events proceed by a mechanism distinct from SSA, such as single-end invasion. These results also indicate that the relative efficiency of the alternative mechanism is inversely proportional to repeat length.

Rad3p has DNA-DNA and DNA-RNA helicase activities. Rad3p is an essential component of the TFIIH transcription initiation factor and it is important for NER. Maines et al. *(109)* isolated an allele of *rad3* called *rad3-G595R* that confers temperature-sensitive growth, a mild reduction in transcription, and increased spontaneous deletions specifically in short direct repeats (<200 bp). *rad3-G595R* also enhances integrative transformation of plasmid DNA sharing limited homology to a chromosomal target. *rad3-G595R* is not UV-sensitive, indicating that NER is functional. Short-repeat recombination can cause significant genome instability even in organisms with relatively little repetitive DNA, such as yeast. The hyper-recombination phenotype of *rad3-G595R* allele is distinct from the *rem* alleles of *RAD3* (Subheading 2.1.) because *rem* only enhances recombination between long regions of homology, such as allelic gene conversion. *rad3-G595R* mutants also display reduced processing at DSB ends. These data were explained by a model in which a helicase defect in *rad3-G595R* reduces end-processing, leading to longer-lived single-stranded tails in short repeats and thereby promoting SSA (or integrative transformation), whereas rapid processing of DSB ends in *RAD3* strains quickly eliminates short repeats and they are unavailable for recombination. Ss11p is another component of TFIIH known to interact with Rad3p. A mutant allele of *SSL1 (ssl1-T242I)* was isolated as a suppressor of *rad3-G595R* temperature sensitivity. Interestingly, *ssl1-T242I* alone mimicked several of the *rad3-G595R* phenotypes, including increased short-repeat recombination, whereas the double mutant had wild-type (low) levels of short-repeat recombination and normal end-processing *(109).* These findings confirm a close association of Rad3p and Ss11p and they provide an interesting example of compensatory mutations in a pair of interacting, multifunctional proteins.

3.2. Recombination in RAD6 (Damage Tolerance) Epistasis Group Mutants

Ty and non-Ty direct repeat recombination and Ty and non-Ty ectopic gene conversion were examined in mutants in the *RAD6* epistasis group *(99). rad5* and *rad18* single mutants had increased levels (3- to 20-fold) of all four types of recombination, and sim-

ilar increases were seen in the *rad5 rad18* double mutant, indicating that these genes are epistatic with respect to spontaneous recombination. Interestingly, these genes also have roles in mutagenesis but they do not show the same epistasis in mutagenesis assays. The high levels of recombination seen in *rad5* and *rad18* are reduced to the (low) *rad1* levels in *rad1 rad5* and *rad1 rad18* double mutants. That a *rad1* mutation can eliminate the increase in gene conversion seen in *rad5* and *rad18* mutants provides additional support for the idea that Rad1p has a direct role in gene conversion distinct from its role (with Rad10p) in processing SSA intermediates.

Rad5p has been implicated in channeling repair of transformed plasmids with a double-strand gap (with noncohesive termini) from NHEJ to a gene-conversion pathway. In *RAD5* cells, 99% of repair led to gene conversion with chromosomal sequences donating information (1% had point mutations). In contrast, only 25% of transformants in *rad5* mutants resulted from gap repair, and 75% resulted from NHEJ, yielding up to 8 bp deletions. These results implicate Rad5p in DNA end-protection or in a more direct (positive) role in homologous recombination *(3).* This transformation system was recently used to test *rad18* and *rad5 rad18* double mutants. *rad18* mutants display essentially 100% gene conversion (much higher than the 25% with *rad5*), although 12% had point mutations outside the gap-repair tract, likely reflecting *rad18*-enhanced mutagenesis. The *rad5 rad18* double mutant displayed an interesting mixture of single mutant phenotypes: gene conversion was intermediate (90%), suggesting that *rad18* suppresses to a large extent the *rad5* defect in gene conversion, and there were few point mutations suggesting that *rad5* suppresses the *rad18* mutagenesis phenotype. As with *rad5,* nonconverted products in the *rad5 rad18* double mutant arose by NHEJ, but deletions were much larger, up to several hundred bp in length (F. Eckardt-Schupp, personal communication). These results suggest that Rad5p and Rad18p may operate together in various DNA repair processes, perhaps in one or more complexes.

Although spontaneous Ty recombination is increased in UV-repair mutants, Ty recombination is minimally enhanced by UV *(96).* It is curious that Ty recombination is refractory to induction by UV because Ty recombination is strongly enhanced by targeted DSBs *(137).*

3.3. Recombination in Checkpoint Mutants

Another class of mutants that are sensitive to UV light are those with defective checkpoints (Vol. 1, Chapter 17), including *dun1, mec1, mec3, rad9, rad17,* and *rad24.* Checkpoint systems are thought to delay cell-cycle progression in response to DNA damage, presumably to allow time for repair before replication or mitosis. If replication of damaged DNA creates recombinogenic lesions (i.e., DSBs), checkpoint mutants would be expected to display hyperrecombination phenotypes, and this is generally true.

Dun1p is a member of a family of protein kinases that includes the checkpoint proteins Rad53p, Mec1p (a relative of mammalian ATM), and Hrr25p. *dun1* mutants display several phenotypes including inability to induce *RNR3* (encoding ribonucleotide reductase) and *MAG1* (encoding a methyladenine DNA glycosylase), a partial G2 checkpoint defect, and increased sensitivity to UV and MMS (Vol. 1, Chapter 18). In addition, *dun1* mutants display increased spontaneous and UV-induced recombination, including gene conversion and SCE *(54);* increased SCE requires replication of DNA containing UV lesions because *dun1* does not increase SCE in G2-arrested cells. It is

possible that the increased spontaneous recombination in *dun1* reflects increased replication of DNA containing endogenous lesions. However, because *dun1* has pleiotropic effects, *dun1* recombination phenotypes might reflect other factors, such as nucleotide imbalance or defective phosphorylation of repair proteins *(54).*

Rad9p controls the G1/S and G2/M checkpoints and it partially controls the S checkpoint. As with *dun1, rad9* mutants display increased spontaneous and UV-induced recombination, measured by reciprocal translocation. However, *rad9* mutation had little or no effect on SCE. It was proposed that Rad9p channels UV and spontaneous damage toward SCE, thus reducing translocations *(53).* Rad24p controls the G1/S, G2/M, and meiosis I checkpoints, and it partially controls the S checkpoint (Vol. 1, Chapter 17). Paulovich et al. *(138)* showed that SCE has different dependencies on Rad9p and Rad24p depending on the status of *RAD1.* Thus, SCE in *rad1* mutants depends in part on Rad9p and Rad24p, but SCE is independent of these genes in *RAD1* cells. A model accounting for these data suggests that *RAD1* cells have two pathways for stimulating SCE: replication past excision-repair tracts forming recombinogenic daughter-strand gaps and replication past unrepaired lesions (perhaps forming DSBs). In *rad1* mutants, UV lesions are not excised, so only the latter pathway is available. Apparently Rad9p and Rad24p play roles in the pathway that processes unrepaired lesions into SCE events, but their precise functions remain unclear.

Mutant *mec1* (also called *esr1*) strains are sensitive to UV and MMS *(88). MEC1* is related to other known checkpoint genes such as *Schizosaccharomyces pombe* rad3$^+$. Interestingly, *mec1* mutation increases mitotic recombination but strongly reduces meiotic recombination; the meiotic phenotype likely reflects, at least in part, the fact that *MEC1* is induced 20-fold during meiosis. These complex phenotypes, as well as the observation that *mec1* null mutants are inviable, argues for numerous roles for Mec1p in both mitosis and meiosis.

3.4. Relationships Among MMR, UV Repair, and UV-Induced Recombination

The MMR system is intricately linked to recombination, and there is increasing evidence for functional overlap between MMR and UV repair. In *E. coli,* UV-induced recombination in UV repair-defective *(uvrA)* mutants is strongly dependent on MMR, and it has been suggested that excision repair and MMR might act in a coordinated manner to form recombinogenic substrates *(55,56).* MMR and NER both recognize several different types of non-B form DNA arising from DNA damage and errors in replication. Mismatches are formed in recombination intermediates during strand invasion, branch migration, and strand annealing ("pairing"), and MMR strongly influences the outcome of recombination events. For example, DSB-induced gene conversion is largely a consequence of MMR of heteroduplex DNA *(140,198).* Spontaneous recombination frequencies are also controlled by the MMR system: levels are substantially reduced by sequence divergence ("homeologous recombination"), but levels are increased to those seen with homologous substrates in MMR-defective strains, such as *msh2* and *msh3* mutants *(129,* and refs. therein).

In mammals and *E. coli,* MMR-defective mutants display modest UV sensitivity. These results are consistent with the findings that TCR of UV damage is absent in *E. coli mutS* and *mutL* mutants, and in human cells with mutations in either *hMLH1* or *hMSH2 (114,115).* Although yeast MMR-defective mutants are not more sensitive to

UV killing than wild-type cells, and there is no dependence of TCR on the MMR system *(171),* UV-sensitivity of yeast NER mutants is increased by an *msh2* mutation *(13).* Furthermore, Msh2p interacts with many NER proteins, including Rad1p, Rad2p, Rad3p, Rad10p, Rad14p, and Rad25p, supporting the idea that MMR and NER proteins exist in complex(es) that may function together in some forms of DNA repair *(13).*

As discussed in Subheading 3.1., Rad1p/10p is important for processing SSA recombination intermediates. Interestingly, Rad1p/10p-dependent processing during SSA also involves the mismatch-recognition proteins Msh2p and Msh3p *(154).* This finding prompted an examination of the effects of UV-repair proteins on spontaneous homeologous recombination between inverted repeats with four heterologies *(129).* In this system, *msh3* and *msh2* mutations increased recombination 8- to 22-fold, respectively, whereas *rad1* and *rad10* mutations increased recombination 6- to 8-fold. The *rad1* and *rad10* effects likely reflect changes in structure-specific processing of recombination intermediates, rather than effects of defective NER, because neither *rad2* nor *rad14* increased homeologous recombination *(129).* These results indicate that Rad1p and Rad10p function with Msh2p and Msh3p in reducing homeologous recombination.

Exo1p is a member of a family of structure-specific nucleases that includes Rad2p (XPG homolog) and Rad27p (flap endonuclease; FEN1); the Exo1p substrate specificity more closely aligns it with Rad27p. *EXO1* is also related to *DIN7* and both are induced by UV. An *exo1 rad2* double mutant is more UV-sensitive than either single mutant, and similar results were obtained when *exo1* was combined with *rad51, rad52,* or *msh2,* suggesting that Exo1p confers resistance to UV independently of NER, recombination, and MMR *(143).* However, Exo1p does have a role in MMR in *S. cerevisiae,* as does exo1$^+$ in *S. pombe (172,182),* and Exo1p interacts with Msh2p *(178).* In *S. cerevisiae, exo1* mutants show reduced spontaneous deletion (SSA) events in direct repeats. When *exo1* was combined with either *rad1* or *rad52,* SSA was not reduced below the levels seen in the single *rad* mutants, indicating that Exo1p acts in the Rad52p- and Rad1p-dependent pathway, at least for deletion in direct repeats *(57).* Exo1p homologs have been identified in *S. cerevisiae, S. pombe,* Drosophila, and humans *(47,57,172,177),* and it is functionally conserved: expression of human *EXO1* complements several yeast *exo1* phenotypes, including UV sensitivity, reduced spontaneous recombination, and increased mutation *(144).* The precise role of Exo1p in recombination is unclear, but it seems likely that it processes DNA ends.

3.5. Recombination Promotes UV Resistance: Roles of PSO4, RAD51, and RPA/RFA1

As discussed in Subheading 2.1., several lines of evidence implicate recombination as a key factor promoting cell survival of UV damage. Additional support for this idea comes from analysis of *PSO4, RAD51,* and replication protein A (RPA). The phenotypes of *pso4* mutants parallel those of *E. coli* recA mutants: both are sensitive to mutagenic chemicals and radiation, and both show defects in induced mutagenesis and recombination. *PSO4* has therefore been assigned to both *RAD6* and *RAD52* epistasis groups. *pso4* mutants display decreased spontaneous allelic and direct-repeat recombination *(4). pso4* mutants are almost completely blocked for UV-induced direct-repeat recombination, with both gene conversion and reciprocal exchange affected *(112).* Together with the UV-sensitivity of *pso4* mutants, these data are consistent with the

idea that UV-resistance is dependent in part on recombinational repair. This idea gains support from several other findings: expression of RecA in yeast increases resistance to UV *(25);* expression of RecA increases UV-induced recombination *(190);* the UV-sensitivity of *rad51* mutants can be suppressed by overexpression of another recombinational repair protein, Rad54p *(36).*

RPA has three evolutionarily conserved subunits of 70, 36, and 14 kDa; the 70 kDa subunit is encoded by *RFA1* and has been subjected to considerable mutant analysis. RPA is involved in DNA replication *(24)* and is an essential component of NER *(72).* RPA has roles in replication initiation and elongation, stabilizing single-stranded DNA (ssDNA) generated at replication forks, and it stimulates DNA polymerase α. *S. cerevisiae RFA1* is an essential gene, consistent with its key role in DNA replication *(24,79).* Many *rfa1* mutants are temperature-sensitive and they display a variety of other phenotypes, including slow growth, UV sensitivity, checkpoint defects, and altered recombination *(26,58,106,107,159,184).* RPA (or the Rfa1p subunit) stimulates strand exchange by Sep1p, enhances homologous pairing and strand exchange by Rad51p, and mediates Rad51p-Rad52p interactions *(79,128,168–170).*

Although many UV-sensitive mutants display increased levels of recombination, *rfa1* mutants have reduced levels of spontaneous allelic and DSB-induced plasmid × chromosome recombination *(58,107).* A comprehensive screen identified 24 *rfa1* mutants, many of which were temperature- and UV-sensitive. Although UV sensitivity might reflect defects in NER, many of these *rfa1* mutants were also sensitive to MMS and HO nuclease, and they had defects in HO nuclease-induced recombination. On the basis of these results it was proposed that the UV sensitivity of these mutants may reflect a defect in DSB repair *(184).* Another *rfa1* allele *(rfa1-D228Y)* was also UV sensitive, but this mutation increased spontaneous direct-repeat recombination. As with other *rfa1* mutants, *rfa1-D228Y* mutants are deficient in spontaneous allelic recombination, and direct-repeat recombination yields principally deletions by a Rad52p-independent mechanism. Thus, *rfa1-D228Y* appears to channel recombination intermediates from a conversion to a nonconversion (Rad52p-independent) pathway *(159).* In this view, the UV sensitivity of *rfa1-D228Y* is consistent with a defect in DSB repair.

4. UV-INDUCED RECOMBINATION IN MAMMALIAN CELLS: *GENETIC CONSEQUENCES*

4.1. UV-Induced Extrachromosomal Recombination

Several strategies have been employed to monitor UV-induced recombination in extrachromosomal DNA substrates. The cells, exogenous DNA, or both can be irradiated and the frequency that stable transfectants are recovered is taken as a measure of illegitimate recombination. By using two inactive copies of a selectable marker, either on separate plasmids or as repeats on a single plasmid, one can monitor extrachromosomal homologous recombination. However, in these assays both homologous and illegitimate recombination must occur to generate a stable transfectant, and it is sometimes difficult to distinguish the effects of UV on these distinct events. Treatment of plasmid DNA with UV prior to transfection enhances transfection efficiency and/or extrachromosomal homologous recombination *(67,78).* UV-enhanced extrachromosomal homologous recombination does not require plasmid replication *(27).* Irradiation of cells

usually enhances transfection, although no effects were seen with some cell types (reviewed in ref. *78).* Thus, UV generally enhances extrachromosomal recombination.

4.2. UV-Induced Recombination Between Chromosomal Repeats

Spontaneously derived duplications in the hypoxanthine phosphoribosyl transferase *(HPRT)* gene were used in several studies to investigate UV-induced homologous recombination in chromosomal repeats. Because *HPRT* is present on the X chromosome, it is hemizygous in male cells and functionally hemizygous in female cells. These *hprt* substrates can form a functional *HPRT* gene upon deletion of the duplicated sequence. Two studies demonstrated that UV doses of 8 to 20 J/m^2 enhanced recombination two- to sixfold above spontaneous levels in an *hprt* gene containing a 13.7 kbp intragenic duplication of exons 2 and 3 *(7,97).* A similar duplication of *HPRT* exon 2 and its flanking regions in a Chinese hamster ovary (CHO) V79 derivative showed a fivefold increase in deletions following a UV dose of 10 J/m^2 *(204).* Another V79-derived cell line with a different *hprt* duplication showed similar levels of UV-induced recombination, although in this case functional *HRPT* genes were thought to arise by illegitimate rather than homologous recombination *(77).* The modest increases in UV-induced recombination seen in these *hprt* studies may reflect, at least in part, high spontaneous reversion frequencies.

More detailed product analysis was possible with a substrate carrying directly repeated copies of the herpes *TK* gene inactivated by *Xho*I linker insertions at different positions and integrated into thymidine kinase-deficient mouse L cells. In these cells, a UV dose of 12 J/m^2 increased recombination 30-fold *(196).* Molecular analysis showed that 85–90% of UV-induced TK$^+$ recombinants arose by gene conversion, and similar results were obtained for spontaneous events *(103).* The remainder were deletions, reflecting intrachromosomal crossovers or unequal SCEs. Gene conversions were also predominant for UV-induced and spontaneous recombination with the same *TK* substrate in a human fibroblast cell line *(14),* with hygromycin direct repeats in another human fibroblast cell line *(183),* and with *neo* direct repeats in a CHO cell line *(43).* In contrast, spontaneous and UV-induced recombination between *neo* inverted repeats yielded relatively few simple gene conversions, with most products displaying complex structures consistent with multiple rearrangements *(43).*

A preliminary analysis with *neo* direct-repeat substrates suggests the conversion: deletion ratio for UV-induced events may be influenced by the number of heterozygosities and by transcription (our unpublished results). In these substrates, the *neo* repeats were 1.4 kbp in length, one *neo* was driven by the dexamethasone-inducible MMTV promoter, and the second lacked a promoter. In a substrate with 13 heterozygosities in *neo* and with low-level transcription of MMTV*neo* (dexamethasone absent), UV-induced predominantly gene conversions. In contrast, with high-level transcription (dexamethasone present), most UV-induced recombinants had deletions (reflecting crossovers, SSA, or unequal SCE). Thus for UV-induced events, transcription levels influence the relative frequencies of gene conversions and deletions. This effect may be specific for multiply heterozygous substrates, because UV induced predominantly gene conversion in a related substrate with a single heterozygosity, regardless of transcription levels *(43).* Because these substrates were integrated at different chromosomal loci, this difference may reflect a position effect; targeted substrates will be required to distin-

guish these possibilities. If multiple heterozygosities promote a deletion mechanism (or inhibit gene conversion) for UV-induced events, this would have important implications with regard to stability of mammalian genomes, which have large amounts of diverged, repetitive sequences.

5. MECHANISM OF UV-INDUCED RECOMBINATION IN MAMMALIAN CELLS

5.1. Stimulation of Recombination by DNA Damage or by Repair?

As discussed in Subheading 3.1., recombination might be stimulated by UV damage per se, or as a consequence of repair processing. This question has been addressed in mammalian cells with defects in NER, and by modulating TCR in recombination substrates with regulated promoters. There is a linear relationship between the extent of UV damage to plasmids and illegitimate recombination as measured by transfection frequency *(165).* However, transfection of NER-deficient XP cells with mutagen-damaged plasmids led to increased transfection compared with repair-proficient cells *(165,186),* indicating that damage-induced illegitimate recombination is not enhanced by NER. A similar conclusion was reached for homologous recombination between chromosomal direct repeats as NER-deficient XP cells required lower doses of UV than NER-proficient cells to reach a particular recombination level *(14,15,183).* SCEs observed cytogenetically arise by homologous recombination *(164).* As with direct-repeat recombination, UV-induced SCE was more frequent in NER-deficient XP cells than NER-proficient cells *(2,40).* Because UV repair can be enhanced by increasing transcription levels, an alternative approach to determine whether recombination is enhanced by UV damage or repair is to modulate repair levels by modulating transcription. The advantage of this approach is that recombination is monitored at a single locus, avoiding problems associated with chromosomal position effects *(18).* The effects of transcription on UV-induced *neo* direct-repeat recombination were monitored in CHO cells, with one *neo* driven by the MMTV promoter. Although transcription and UV separately stimulated recombination, increasing transcription levels enhanced TCR and this reduced UV-induced recombination. Together these studies indicate that DNA damage, not repair by NER/TCR, stimulates illegitimate and homologous recombination. There are at least three other repair pathways (photolyase, glycosylases, UVDE) (reviewed in ref. *203).* However, only a small fraction of UV lesions are processed by these alternative repair pathways, and it is unlikely that they significantly impact UV-induced recombination.

A corollary to the idea that recombination is stimulated by UV damage and not repair is that poorly repaired regions may be recombination hotspots. Repair of dimers can vary between genes, bulk chromatin or even between neighboring base positions *(66,180).* For example, along the *p53* gene, sites of skin-cancer mutation hotspots are almost always sites where DNA repair processing is particularly slow *(180).* These sites may have a higher probability for initiating recombination events.

As discussed in Subheading 3.4., there is significant overlap between the NER and MMR systems. Mutations in the human MMR genes *hMSH2* and *hPMS2* render cells slightly more sensitive to UV *(114,115).* Active MMR was not required for spontaneous or UV-induced SCE in a human cell line, indicating that SCE can occur independently

of lesion recognition/processing by MMR *(132).* This is consistent with the report that purified hMutSα (MSH2-MSH6) mismatch recognition complex does not recognize UV-induced DNA photoproducts *(125).* However, hMutSα does recognize compound UV/mismatch lesions *(193),* which are thought to arise during error-prone translesion synthesis *(63).* Compound lesions are removed more efficiently by excision repair and recognized less efficiently by the MMR system *(125).* Compound lesions are highly mutagenic but they do not appear to be sites of recombination *(193).* Thus, neither NER nor MMR is required for UV-induced recombination in mammalian cells.

5.2. Does Transcription Stalling at UV Lesions or Gene Induction Enhance Recombination?

It is well-established that bulky DNA lesions such as CPDs block transcription both in vivo and in vitro *(48,155).* The mRNA levels of genes required for recombination might be reduced if UV lesions blocked transcription in these genes and this could presumably reduce the level of recombination. However, such effects would be expected only at very high doses where a significant fraction of cells suffered damage to genes required for recombination, and only if recombination proteins had short half-lives relative to the rate of repair.

UV induces many genes in mammalian cells *(5,44,46,80,108),* including two in humans with important roles in homologous recombination, *hRAD51* and *hRAD51B (139).* Overexpression of *hRAD51* in human cells increases spontaneous recombination *(200).* Thus, a fraction of UV-induced recombination in mammalian cells may occur at undamaged loci owing to enhanced expression of *RAD51* and other recombination genes. Although there is clear evidence for indirect stimulation of recombination by UV in yeast (Subheading 2.2.), analogous cell-fusion experiments have not been performed in mammalian cells.

5.3. Roles of Replication, PCNA, BRCA1, BRCA2, RAD51, and RPA in UV-Induced Recombination

There is no direct evidence linking replication-fork arrest at UV lesions with recombination in mammalian cells, but indirect evidence suggests a probable connection. Because replication-fork pausing is conserved from bacteria to humans, it seems likely that the mechanisms for processing stalled or arrested forks are also conserved and tightly regulated. Although UV lesions cause significant helix distortion *(92,181),* DNA replication inhibition and cell-cycle arrest does not reflect a direct block by these helix-distorting lesions, but an indirect effect mediated by increased levels of $p21^{Cip1/WAF1}$ *(31,49,195),* an inhibitor of cyclin-dependent kinases *(141).* Thus, in mammalian cells as in yeast, replication of damaged DNA may produce DSBs, and in fact DSBs have been observed following UV exposure of mammalian cells *(194).*

Several proteins have been implicated in UV-induced recombination in mammalian cells, including proliferating cell nuclear antigen (PCNA), BRCA1, BRCA2, RPA, RAD51, and RAD54. PCNA is a replication processivity factor that is essential for DNA replication and cell-cycle arrest, BER, and MMR *(70,111,160,185).* PCNA appears to localize at sites of replication during S phase *(22,23).* Although the interaction between p21 and PCNA inhibits PCNA function in S-phase replication, it does not affect PCNA function in DNA synthesis during repair *(60,98,191).* Germline mutations

in the breast-cancer susceptibility genes, *BRCA1* and *BRCA2,* predispose women to early onset of familial breast and ovarian cancers *(11,35,197).* Both genes are now implicated in recombination (Chapter 10); in particular, *BRCA1* is important for DSB-induced homologous recombination *(123).* In yeast and higher eukaryotes, RAD51 forms nucleoprotein filaments on ssDNA, mediating homologous pairing and strand-exchange reactions *(9,10,71,168).* In human cells, RAD51 nuclear foci increase after UV irradiation; these foci probably represent sites of recombinational repair *(74).* Upon UV irradiation of S-phase MCF7 cells, BRCA1 was found to be phosphorylated and co-localized with PCNA and RAD51 *(156).* A subsequent study showed that BRCA2 is also co-localized with PCNA *(32).* The interactions between BRCA1, BRCA2, and RAD51 and their recruitment to replication forks after UV exposure *(32–34,156)* strongly implicate these proteins in UV-induced recombination.

As in yeast, mammalian RPA is involved in DNA replication *(90),* NER *(124),* and recombinational repair. RPA has a role in both initiation and elongation of replication, stabilizing ssDNA generated at replication forks and stimulating DNA polymerase α activity *(24,89).* In human cells, UV-induced inhibition of replication can be reversed by the addition of RPA, suggesting a role for RPA in this regulatory event *(31).* RPA interacts with RAD52 and this interaction appears to be required for homologous recombination in mammalian cells *(136).* DNA damage causes RAD51 and RPA to form specific foci at ssDNA sites, suggesting that these are sites of recombinational DNA repair *(145).* The limited data in mammalian cells is consistent with the view developed from yeast studies that UV-induced recombination involves an interplay among replication functions (PCNA, RPA) and recombination functions (RAD51, RAD52, BRCA1, BRCA2).

The 70 kDa subunit of RPA interacts with the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) *(158),* and DNA-PKcs is involved in UV-induced replication arrest through modulation of RPA activity *(135).* DNA-PK is a nuclear serine/threonine protein kinase consisting of DNA-PKcs and the Ku heterodimer (Ku70 and Ku80) and this complex has an integral role in NHEJ (Vol. 2, Chapter 16). Thus, the interaction between RPA and DNA-PK might be important for UV-induced illegitimate recombination.

5.4. Negative Regulators of UV-Induced Recombination: p53 and XRCC9

Certain biological effects of UV irradiation are expected to reduce levels of recombination. p53 is induced by UV light, and it has roles in DNA repair, checkpoint control, and apoptosis *(76,161).* Cells lacking p53 or with mutated p53 have higher rates of homologous recombination than wild-type cells *(12,113,167,199).* Therefore UV-induction of p53 might be expected to downregulate recombination. Extrachromosomal recombination induced by site-directed psoralen adducts was similar in cells with wild-type p53 and cells with p53 inactivated by E6 protein *(52).* To date, a direct comparison of UV-induced recombination levels in wild-type and p53 mutant cells has not been made. The ATM protein is thought to function in the same pathway as p53 in G1/S checkpoint control (Vol. 2, Chapter 19), and *atm* mutants also display increased levels of spontaneous homologous recombination *(16,117).* It is possible that the increased spontaneous recombination in *p53* and *atm* mutants is a consequence of the G1/S checkpoint defect, which increases replication of spontaneous damage, as hypothesized for yeast checkpoint mutants (Subheading 3.3.).

The CHO mutant UV40 is hypersensitive to UV but NER-proficient, and UV40 cells are defective in expression of *XRCC9 (105).* UV40 cells show high levels of SCE, elevated chromosomal instability, and increased sensitivity to UV inhibition of replication *(28). XRCC9* was subsequently shown to be homologous to the gene defective in Fanconi anemia group G patients, *FANCG (41).* As with UV40, *FANCG* mutant cells show chromosome instability. Thus, XRCC9, like p53, appears to negatively regulate recombinational repair processes. Observed levels of UV-induced recombination likely reflect a balance between recombination suppression, which preserves gross genomic integrity, and recombination enhancement, which promotes DNA repair and/or recombinational restart of collapsed replication forks.

6. DNA-REPAIR PATHWAYS, DELAYED GENOMIC INSTABILITY, AND CANCER

It is becoming clear that there is considerable overlap among various DNA-repair pathways. The similar effect of *msh2, msh3, rad1,* and *rad10* on homeologous recombination (Subheading 3.4.) provides a striking example of overlap between MMR and UV-repair pathways (reviewed in ref. *151).* Cellular responses to DNA-damaging agents reflect complex networks that operate from the nucleotide to the chromosome and whole-cell levels, including repair systems such as NER, and tolerance systems such as translesion synthesis, lesion bypass via recombination, and checkpoints. In mammalian cells, these networks produce a balance between repair of DNA damage and cell death by apoptosis. It is now well-established that defects in NER increase spontaneous and UV-induced mutagenesis and recombination. Cancer predisposition associated with NER defects may result both from enhanced mutagenic and recombinogenic effects of spontaneous and UV-induced DNA damage.

Genomic instability is a hallmark of cancer. Instability is expressed in many ways, including chromosome rearrangement, aneuploidy, gene amplification, increased mutation rate, and increased instability of short repeated sequences such as micro- and minisatellites *(20,69,100,122,126,134,142).* Cells displaying one type of instability may or may not display other types. Thus, instability may result from dysregulation of any of a number of "stability functions." It has long been known that DNA damage caused by radiation can be converted rapidly to mutations and chromosome aberrations. It recent years it has become apparent that radiation also has delayed effects, inducing genomic instability and/or mutations many generations after exposure. Although most research on delayed instability has focused on the effects of ionizing radiation *(104,122,126),* an early report indicated that UV can induce delayed chromosome aberrations *(82)* and there is evidence that UV induces a delayed mutator phenotype *(166).* At least for ionizing radiation, delayed effects appear to reflect epigenetic changes, rather than direct genetic effects *(127).* It is possible that epigenetic changes are responsible for the indirect stimulation of recombination by UV seen in *S. cerevisiae* (Subheading 2.2.). UV is a complete carcinogen and it is reasonable to suspect that this is at least part owing to its direct mutagenic effects. With our better understanding of the broader effects of UV, a central question is whether indirect effects, such as induced mutator and chromosome instability phenotypes, also contribute to UV carcinogenesis.

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14

Telomeres, DNA Repair Proteins, and Making Ends Meet

Susan M. Bailey, Julianne Meyne, and Edwin H. Goodwin

1. INTRODUCTION

Telomeres are unique structures at the physical ends of linear eukaryotic chromosomes. They were first described over 60 years ago by Hermann Muller in his classic studies of the fruit fly *Drosophilia melanogaster (78).* He coined the name 'telomere' from the Greek—*telos* meaning end and *meros* meaning part—based on their chromosome end protection function. Shortly thereafter, Barbara McClintock's cytogenetic observations in maize demonstrated that broken chromosomes could fuse with one another to form dicentric chromosomes *(72,73).* These early studies brought to light the fact that natural chromosome ends are distinguished from random breaks and protected from illegitimate end-joining reactions. How the cell accomplishes this critical discrimination is still under investigation.

Telomeres continue to hold scientist's fascination today, particularly as new cytogenetic and molecular biology technologies have opened additional doors of understanding into their structure and function. Amazingly, telomeres have been found to be involved not only in chromosome stability, but also in chromosome replication, nuclear architecture, gene expression, human tumor formation, aging, and senescence *(118).* Most recently, and perhaps most surprisingly, DNA repair proteins have been discovered to play an essential role in the normal end-capping function of chromosomal termini.

2. TELOMERE BIOLOGY

Telomeric DNA consists of tandem arrays of short, repetitive G-rich sequences that are oriented 5′-to-3′ towards the end of the chromosome *(7,9),* forming a 3′ single-stranded G-rich overhang *(66,113).* Together with an ever-increasing number of known telomeric binding proteins, a dynamic terminal structure is created that "caps" both ends of linear chromosomal DNA molecules and provides protection from exonucleolytic attack and degradation, as well as preserves genomic stability by preventing undesired end-joining reactions *(10,88,119,120).* Because there are no genes contained in repetitive DNA, it has been thought of as "junk" DNA. However, as can easily be

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seen from the vital functions that telomeres perform, this way of thinking about these special regions of the genome requires reexamination.

2.1. Telomere Replication

The importance of telomeres to the cell is evidenced by the fact that functional telomeres are essential for continuous cellular proliferation, an observation that has profound implications in our understanding of aging and cancer *(48,49,77).* Although telomeres are vital for chromosomal stability and cell survival, telomere maintenance presents several unique problems to the cell. First, telomeres are faced with an end-replication problem. Because conventional DNA polymerases replicate only in the 5′-to-3′ direction, can only extend existing DNA or RNA chains, and need a complementary strand to provide a template, they are unable to replicate to the very end of a linear duplex *(83,111).* During semiconservative DNA replication, short RNA primers (8–12 bp) made by RNA primase are required to initiate lagging-strand replication, which are then extended by DNA polymerase to form Okazaki fragments. As these RNA primers are removed and fragments ligated together, there are no means to synthesize the lagging-strand sequence complementary to the short region at the very end of a chromosome, so a gap results. It has also been proposed that the end replication problem is a result of the inability of leading-strand synthesis to produce a 3′ overhang *(65).* In this scenario, a 5′-to-3′ exonuclease is required to recreate the 3′ overhang after replication, causing telomere strands duplicated by leading-strand synthesis to be shorter than the parental telomere that served as a template during replication *(Fig. 1).* According to a revised model of telomere replication *(66,112),* DNA is lost from both ends of the chromosomes, due to degradation of the 5′ C-rich strand by an S-phase-specific exonuclease activity, resulting in long 3′ overhangs at both ends. Others demonstrate that telomeres generated by leading- versus lagging-strand DNA synthesis differ and suggest that each chromosome has one telomere with a long G-rich overhang (200 ± 75-nucleotide) and one that is either blunt-ended or has a short G-rich overhang (≤12 nucleotides) *(116).* By whichever mechanism, without some type of compensatory mechanism, it is certain that with each cell division telomeric sequence is lost. The average rate of loss in mammalian cells has been estimated to be between 50 and 75 bp/telomere/cell cycle *(89,116).* It also appears that the rate of loss is not constant, but rather fluctuates greatly from cell cycle to cell cycle *(79).*

In germ line cells (and a majority of tumors), the specialized ribonucleoprotein telomerase compensates for telomeric repeat loss *(37).* Telomerase contains an internal RNA template for the 5′-to-3′ addition of TTAGGG repeats to extend the single-stranded G-rich strand of the chromosome end *(38).* However, in most adult human somatic cells telomerase is inactive or present at very low levels *(55).* Hayflick recognized in the 1960s that normal cells undergo a finite number of doublings in cell culture *(50).* It has since been suggested that the progressive erosion of telomeres may be responsible for placing an upper limit on the proliferative capacity of somatic cells. According to this hypothesis, once a critically shortened length is reached, the cell no longer recognizes the telomere as a natural chromosome end. Instead, critically shortened telomeres are misidentified by DNA surveillance enzymes as ends created by double-strand breaks, an event that triggers cell-cycle arrest. Tumor cells are thought to overcome this barrier by reactivating telomerase, thereby maintaining their telomeres

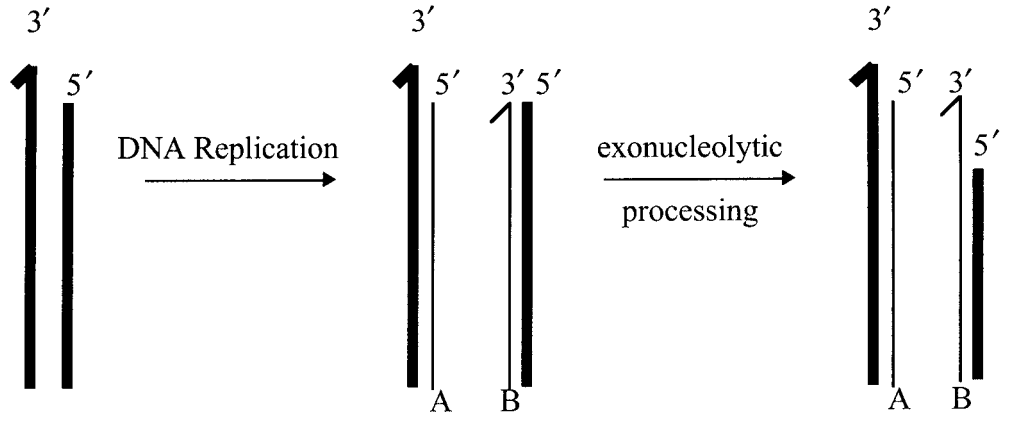


Fig. 1. Mechanism of telomere shortening. The parental 3′ and 5′ telomere DNA strands, shown here as dark lines, are replicated by lagging and leading strand synthesis respectively (newly synthesized DNA is shown by lighter lines). The telomere replicated by lagging strand synthesis *(A)* has a 3′ overhang because this mode of replication can not proceed to the very end of linear DNA. However, this may not necessarily indicate that this telomere has shortened because the parental telomere also had a 3′ overhang. The telomere replicated by leading strand synthesis, *(B)* is initially blunt-ended and requires exonucleolytic processing of the 5′ strand to recreate a 3′ overhang. This processed 5′ strand will then serve as a shorter template for replication in the following cell cycle. Therefore, in this hypothetical mechanism, exonucleolytic processing of the telomere replicated by leading strand synthesis is the source of telomere shortening.

and avoiding senescence *(1,20).* In addition, telomerase-independent pathways, or alternative lengthening of telomeres (ALT), have been proposed as a means to maintain telomere length *(8,18).* These presumably recombination-based mechanisms use telomeric DNA on other chromosomes as a template for extending telomeres. Two such possible mechanisms are illustrated in Fig. 2.

2.2. Telomere-Associated Proteins

Two human telomere-specific DNA binding proteins, TRF1 (TTAGGG repeat binding factor 1) and TRF2 have been identified *(15).* TRF1 is a negative regulator of telomerase activity at chromosomal termini and thus of telomere length, i.e., overexpression of TRF1 leads to progressive telomere shortening, while inhibition increases telomere length *(108).* TRF2 has also been shown to be a negative regulator of telomere length *(101),* as well as protecting chromosome ends from end-to-end fusion events *(109).* TRF2 is the first telomere-associated protein implicated in the maintenance of the correct terminal DNA structure necessary for proper telomere function. A clue as to what that physical structure might be was uncovered by the discovery that TRF2 can remodel mammalian telomeric DNA into large duplex loops, termed t loops *(39).* A t loop is created when a telomere end loops back and the single-stranded G-rich tail invades an interior segment of duplex telomeric DNA. TRF1, which can induce bending, looping, and pairing of duplex DNA *(5,6),* and TRF2, which can induce invasion of the 3′ single-stranded tail into duplex telomeric DNA *(39),* bind duplex telomeric DNA in vivo and both appear to be involved in the formation of t loops *(101).* By sequestering telomeric

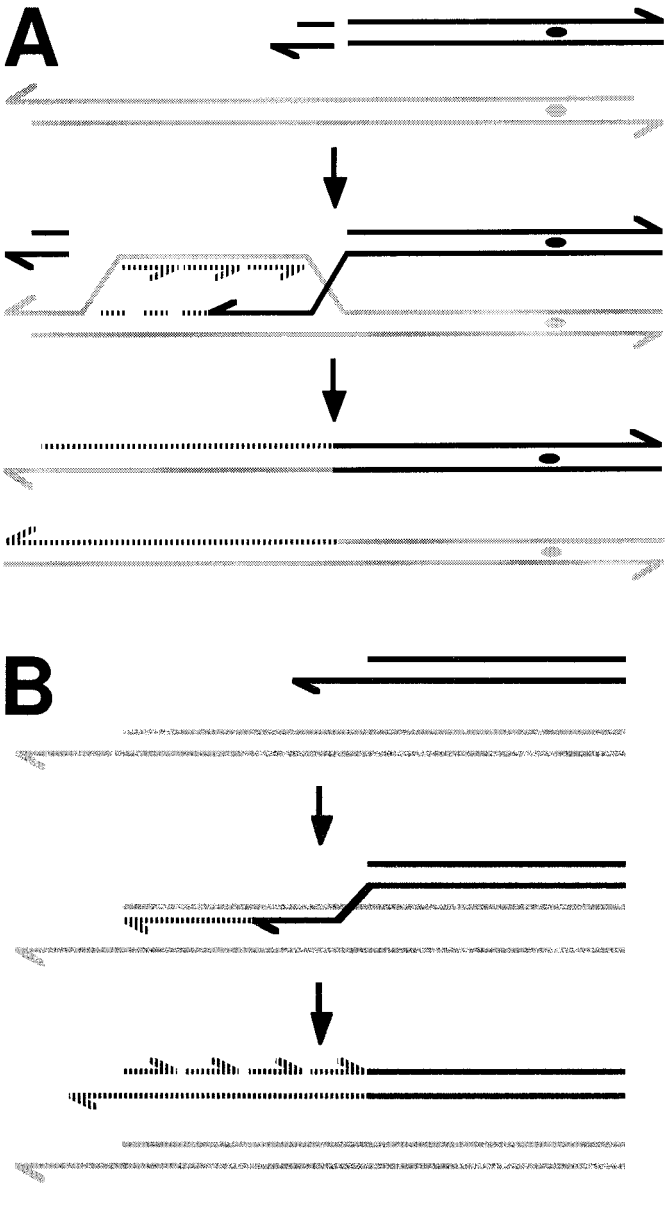


Fig. 2. (A) Telomere elongation by semiconservative DNA synthesis *(43).* Strand invasion by the 3′ terminus of one strand from the centromeric side of a DSB in the dark chromosome initiates leading-strand DNA synthesis in the telomere of the lighter chromosome. Lagging-strand synthesis occurs in the displaced strand (indicated by dashed lines). Following completion of DNA synthesis, the intermediate structure is resolved. The distal end of the DSB is either degraded or engages in unproductive recombination with another target. **(B)** Telomere elongation by conservative DNA synthesis *(118).* Strand invasion of a template telomere initiates leading-strand DNA synthesis. The intermediate structure is resolved and the complementary strand is synthesized by either lagging-strand synthesis or is primed by a terminal hairpin created by the G-rich strand folding back on itself.

termini, t loops may effectively conceal natural chromosome ends from the cell's DNA repair machinery.

TIN2, a TRF1-interacting protein that co-localizes with TRF1 on human metaphase chromosome termini, has also been identified *(56).* Expression of mutant TIN2 causes elongation of telomeres in a telomerase-dependent manner, suggesting that TIN2 mediates TRF1 function and thereby negatively regulates telomere length. Tankyrase, another TRF1-interacting protein, is discussed in Subheading 3.4. Additional telomere-associated proteins with roles in DNA repair are discussed in Subheading 3.

3. DNA REPAIR PROTEINS AND TELOMERE FUNCTION

DNA double-strand breaks (DSBs) represent a major threat to the integrity of the genome because of their potential for causing lethality, mutagenesis, and carcinogenesis if they are left unrepaired or are misrepaired. Many exogenous agents, including ionizing radiation and a number of anticancer drugs (e.g., bleomycin) cause DSBs, as do endogenous free radicals—the natural by-products of oxidative metabolism. DSBs also occur normally as intermediates in site-specific V(D)J recombination, the process that helps to generate the wide variety of antigen-binding sites necessary for antibody and T-cell receptor proteins during lymphocyte development. Consequently, all cell types possess multiple, as well as very effective, mechanisms for the repair of DSBs *(52,121).*

3.1. Modes of DSB Repair

Eukaryotic cells accomplish DNA DSB-repair via at least two pathways, homologous recombination (HR) and nonhomologous (or illegitimate) end joining (NHEJ) *(54,43).* In yeast, the predominant DSB-repair pathway is HR, which involves the exchange of genetic information between a damaged chromosome and an undamaged homolog, sister chromatid, or other region of shared homology. Repair of a DNA DSB by HR occurs by means of replication, using the homologous strand as a template, so typically there is no loss of genetic information. Although the significance of HR in mammalian cells is emerging *(64,105),* currently the majority of DSBs in mammalian cells appear to be repaired by NHEJ, a process which requires little or no homology between the two recombining molecules *(104),* but usually alters the DNA sequence at the point of joining (small deletions or insertions). NHEJ proteins have been shown to be critical for maintaining mammalian genomic stability *(31).* Yeast also possess a NHEJ pathway, which is especially important in haploid G1 phase cells since no homolog is present *(96).* Crucial components of the NHEJ DNA repair pathway appear to be conserved between yeast and mammalian systems *(23).* For example, *Saccharomyces cerevisiae* homologues of both human Ku70 (yKu70 or Hdf1) and Ku80 (yKu80 or Hdf2) have been identified and found to play important roles in NHEJ *(13,14,29,30,53).* One notable exception, the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs), appears to be restricted to higher eukaryotes, as a yeast homologue has not been identified.

Human Ku was originally identified as an autoimmune antigen in patients with polymyositis-scleroderma overlap syndrome *(76).* Ku is the most abundant DNA end-binding protein in both mammalian and yeast cells, and it recognizes many DNA structures in a DNA-sequence-independent manner. Ku is a heterodimer composed of 70 and 86 kDa subunits, which are encoded by the *XRCC6* and *XRCC5* genes, respectively *(28).*

Ku binds with high affinity to double-stranded DNA ends, whether blunt, overhanging or hairpin in structure *(27).* Mutant cells lacking Ku are deficient in the repair of DSBs, as well as in recombination of the immunoglobulin V(D)J region, and are hypersensitive to radiation *(41),* yet the precise role of Ku in DNA double-strand break repair remains elusive. Ku may function as a DNA damage sensor, rapidly identifying and binding to broken ends, then recruiting, or targeting, other proteins to the site. Once bound, Ku could signal the presence of DNA damage through its association with the ~465 kDa catalytic subunit of DNA-dependent protein kinase (DNA-PKcs), which together with the Ku heterodimer comprise DNA-PK, a nuclear serine/threonine kinase *(17,99).* The kinase activity of DNA-PKcs is activated when complexed with DNA-bound Ku *(34).* DNA-PKcs has also been shown to bind DNA itself (i.e., without Ku) but have limited protein kinase activity *(45).* Enlightening structural analysis of DNA-PKcs suggests that activation of the kinase requires interactions with both double- and single-stranded DNA *(46,62).* It becomes interesting and perhaps most important to recognize that only very specific types of DNA structure mediate DNA-PKcs activation. Ku therefore, may serve to stabilize the interaction between the free DNA ends and the catalytic subunit, provide protection from degradation and/or contribute to, or control the chromatin structure necessary for the assembly of a functional Ku/DNA-PKcs/XRCC4/Ligase IV NHEJ repair complex *(90).*

XRCC4 (X-ray repair cross-complementing gene 4) *(63)* encodes a small nuclear phosphoprotein of 334 amino acids, is an effective substrate for DNA-PK *in vitro,* and has been shown to be tightly associated with DNA Ligase IV *(22),* a component necessary for the final step of rejoining broken chromosomal ends *(115).* DNA Ligase IV is also a specific *in vitro* substrate of DNA-PK *(57).* An important role for *XRCC4* in normal development and suppression of tumorigenesis has been demonstrated *(32).*

The Mre11/Rad50/Xrs2 (yeast) complex also functions in DSB repair by both HR and NHEJ *(42),* but its precise role is unclear. It has been demonstrated that the Mre11/Rad50 complex possesses both endonuclease and 3′-to-5′ exonuclease activities *(106),* and so it may process, or prepare, the broken DNA ends for ligation, or it may play more of a structural role *(82).* Well-conserved human homolog of RAD50 and MRE11 have been identified and appear to be physically associated *(26,85).* A complex consisting of human RAD50, MRE11, and another protein of about 95 kDa (p95), has been purified *(106).* The p95 protein, termed Nibrin *(110),* was found to be mutated in Nijmegen Breakage Syndrome (NBS). Nibrin deficiency leads to the chromosomal instability, radiation sensitivity, and cancer predisposition seen in patients with this rare autosomal recessive disorder. Nibrin, encoded by the *NBS1* gene, may be a functional homolog of yeast Xrs2. The function of Nibrin is currently unclear, but p53 upregulation in response to irradiation is abrogated in NBS cells, suggesting a role in DNA-damage signaling and cell cycle checkpoint control *(16).* It is interesting to note that human MRE11 and RAD50 proteins form discrete nuclear foci at sites of damage in response to DSB-inducing agents. These foci do not form in NBS cells, suggesting that Nibrin is required for the localization and/or stabilization of this complex at DSBs *(71).*

Nanoelectrospray tandem mass spectrometry, protein blotting, and indirect immunofluorescence studies have revealed a cell-cycle-regulated association of RAD50/MRE11/NBS1 with TRF2 at human telomeres *(122).* RAD50 and MRE11 co-localized with TRF1 and TRF2 and were present at most interphase telomeres. NBS1 was associated with TRF2 and telomeres only in S-phase and only in a minority of the cells. The

presence of the RAD50/MRE11/NBS1 complex at human telomeres and its association with TRF2 supports a role in mammalian telomere function, perhaps in t loop formation. For additional details about the MRE11/RAD50/NBS1 complex, *see* Chapter 7.

3.2. DSB Repair and Chromosome Instability

Structural chromosomal aberrations are a conspicuous visual manifestation of the misrepair or nonrepair of DSBs *(19).* They can be separated into two distinct classes, chromosome-type aberrations that involve both chromatids of a mitotic chromosome and chromatid-type aberrations, which involve only one chromatid. Aberrations can be classified further based on the unique structures that are formed. Examples include dicentric chromosomes that by definition have two centromeres, acentric fragments having no centromere, ring chromosomes, inversions where a chromosomal segment has become detached and then reintegrated into the same chromosome in an inverted position, translocations created by the exchange of pieces of broken chromosomes, and terminal deletions that are created by unrejoined DSB. Robertsonian translocations are a particular type of exchange that deserve special mention both because they are widespread in mammals and because they will become important in the discussion that follows. Robertsonian translocations are created by the joining of two acrocentric (centromere at one end) chromosomes at their centromeres to form a metacentric (centromere at the middle) chromosome *(91).* In mouse, it has been shown that the p-arm (short-arm) telomeres are deleted when acrocentric chromosomes enter into Robertsonian rearrangements *(33,80,97).*

The consequence for the cell varies depending on the type of chromosomal aberration present. For example, the two centromeres of a dicentric chromosome an asymmetrical exchange, may be pulled towards opposite poles during anaphase resulting in mechanical difficulty in the separation of daughter cells, an event that is often lethal. Acentric fragments also do not segregate properly during mitosis since they do not have a centromere. Some daughter cells consequently suffer the loss of genetic material that may unmask recessive lethal mutations. In contrast, symmetrical exchanges like inversions and translocations are rarely lethal, but may cause mutation of genes located at the breakpoints involved in the rearrangement. Cancer cells frequently contain very specific inversions or translocations, having oncogenes or tumor suppressor genes located at the breakpoints *(102).* Observations such as these illustrate nicely the importance of effective DSB repair in maintaining a stable genetic inheritance.

As the physical ends of linear DNA duplexes, telomeres run the risk of being misidentified as double-strand breaks in need of repair and ligation. The cell must be able to distinguish between natural chromosome ends (telomeres) and broken double strands, as the consequences of illegitimate telomeric end joining, e.g., dicentric chromosomes and Robertsonian translocations, would be disastrous to the cell. With this in mind, recent evidence (primarily in *S. cerevisiae)* that surprisingly locates several proteins of the DNA repair arsenal at telomeres, presents an intriguing paradox. Why are DNA repair proteins, whose function is to bind and join double-stranded ends, present at the telomere where fusion is undoubtedly to be avoided, and what role(s) do they play in normal telomere function?

3.3. DNA Repair Proteins in Yeast Telomere Biology

In addition to functioning in the vital processes of DNA repair, NHEJ proteins have also been found to be required for normal telomere structure and function, maintenance,

and telomere-associated transcriptional silencing *(12).* In vivo crosslinking experiments have demonstrated that the Ku heterodimer binds yeast telomeres *(36).* The absence of the Ku heterodimer affects the perinuclear clustering of telomeres normally seen in wild-type yeast cells, indicating that Ku is required to establish the normal structural arrangement of telomeres in interphase nuclei *(59).* It has also been proposed that Ku protects the telomere from nucleolytic and recombinational activities *(86).* Furthermore, yeast strains defective in Ku70 or Ku80 lose the majority, but not all, of their terminal telomere repeats *(13,87).* Disruption of the *RAD50, MRE11,* or *XRS2* genes also leads to telomere shortening *(3,81).* The roles these proteins play in telomere maintenance and function is currently unclear. The idea that DNA repair proteins may contribute to a terminal end-binding complex that in turn recruits still other proteins necessary for normal telomere function is supported by the finding that yKu70p interacts with Sir4p *(107),* which in turn interacts with Rap1p *(67),* a key regulator of telomere length.

Sir2p, Sir3p, and Sir4p also interact with histones H3 and H4 and function in transcriptional silencing at telomeres by inducing a condensed, inaccessible heterochromatic state in the vicinity of the telomere. As a result, genes at or near telomeres are subject to transcriptional repression, a phenomenon termed telomere position effect (TPE) *(35).* Disruption of Ku debilitates this telomere-associated silencing *(44).* Mutation of *Sir* genes leads to decreased Ku-dependent NHEJ, indicating that in yeast these silencing proteins may also play a role in NHEJ *(60).* In addition, redistribution of yeast telomeric Ku and Sir proteins in response to DNA strand breaks has been demonstrated *(70,75).* These results suggest that Ku recruits the Sir protein complex to sites of DNA damage, inducing a heterochromatin-like state around the broken ends, perhaps through an interaction with nucleosomal DNA *(40).* Nucleases and recombination enzymes may then be excluded from the broken ends, so that degradation and undesirable recombination, such as joining reactions with other DNA ends, are avoided. A model of telomeric silencing might also involve rapid binding of Ku to telomeric DNA and interaction with the Sir protein complex through Sir4p. The resulting chromatin condensation then serves to both repress transcription and protect chromosome ends from degradation and recombination reactions *(107).*

3.4. DNA Repair Proteins in Mammalian Telomere Biology

Telomeres in both yeast and mammalian cells are tandem arrays of G-rich repetitive DNA, but it is important to recognize that differences also exist between the two. Yeast telomeres each consist of ~250–350 base pairs (bp) of the variable TG_{1-3} sequence *(95),* while each mammalian telomere consists of ~5–10 kilo base pairs (kb) in human *(68,69)* to ~50–100 kb in mouse *(58,123)* of the TTAGGG repeat sequence. Mammalian telomeres end in long (130–270 bp) single-stranded G-rich overhangs *(66,116),* whereas long single-stranded overhangs occur at yeast telomeres only briefly in late S-phase *(25,114),* otherwise, yeast telomeres have only an ~10 nucleotide G-rich overhang *(120).* Therefore, although there are similarities, it is not unreasonable to expect differences in the requirements for telomere end-binding activities and protection to exist between the two organisms.

Also in contrast to yeast, the study of DNA repair proteins in mammalian telomere biology has focused primarily on illegitimate recombination events that are observable as telomeric associations and chromosomal end fusions. Chromosomal end-to-end

fusion has been attributed to a loss of telomere function caused by telomere shortening *(21).* In agreement with this hypothesis, the numbers of end-to-end associations have been observed to increase as telomere length decreased over several generations in mouse cells lacking the telomerase RNA subunit (mTR) *(11).* However, analysis of two human cell lines with severely shortened telomeres revealed that they were not detectably compromised in end-capping function *(93).* These results suggest that telomeric length is not the only factor that determines the fusigenic behavior of chromosome ends. In addition, no clear link between short telomeres and fusigenic potential could be demonstrated in murine severe combined immunodeficiency *(scid)* and Chinese hamster ovary (CHO) immortalized cell lines, leading to the speculation that chromosomal fusion may be caused by abnormalities in the structure of telomeric chromatin (98). Interestingly, measurement of telomere lengths in four different *scid* mice by Southern blot analysis of terminal restriction fragments (TRF) resolved on pulsed-field gels, as well as by quantitative fluorescence *in situ* hybridization (Q-FISH) analysis, revealed that *scid* mice consistently had longer telomeres than isogenic wild-type control animals *(47).* Measurements of telomere length in wild-type and Ku80 deficient mice have also revealed that, in contrast to yeast, Ku deficiency does not result in telomere shortening, and in fact telomeres are moderately elongated in Ku mutant cell lines *(94).*

Chromosomal end-to-end associations have been observed in tumor cells and in several human diseases including ataxia telangiectasia (AT). AT is an inherited autosomal recessive disorder featuring chromosomal instability and a high incidence of cancer (*see* Vol. II, Chapter 19). The ataxia telangiectasia mutated (ATM) gene product is defective in AT and is a member of the phosphatidylinositol 3-kinase superfamily, as is DNA-PK and the yeast Tel1 protein. It has been reported that telomeres in normal diploid and Epstein-Barr virus-transformed AT fibroblasts are shorter than in repair-proficient human fibroblasts *(84,117)* and that telomeres shorten at an accelerated rate in AT patients *(74).* However, a separate and more extensive study concluded that SV-40 transformed AT cells did not have a defect in telomere maintenance *(103).*

In mammalian cells, the effectiveness of telomeric end capping can be evaluated with fluorescent *in situ* hybridization (FISH). FISH has proven to be a powerful cytogenetic tool that reveals the chromosomal location of DNA target sequences with homology to fluorescently labeled nucleic acid probes. Spontaneously arising chromosome aberrations can be inspected for telomere signal at the point of fusion between the two chromosomes, the presence of which would give an unambiguous indication that telomeric end capping has failed. Such a strategy was recently employed to assess the ability of normal and DNA repair-deficient mouse cell lines to cap and protect chromosome ends *(2).* Telomeric fusions were not observed in any of the repair-proficient control cell lines examined. However, spontaneously arising fusions in which telomere sequence was retained at the points of joining were observed in mutant cell lines deficient in either Ku70, Ku80, or DNA-PKcs, clearly indicating that telomeric end-capping had failed in these mutants *(Fig. 3).* A mutant cell line deficient in p53 was also examined, but telomere-positive fusions were not observed at levels significantly above wild type controls. DNA-PKcs-deficient *scid* cells were analyzed in both low passage primary cultures and again after spontaneous immortalization. One type of fusion product, telomere-positive Robertsonian translocations, became especially prevalent after immortalization and their numbers continued to increase with further

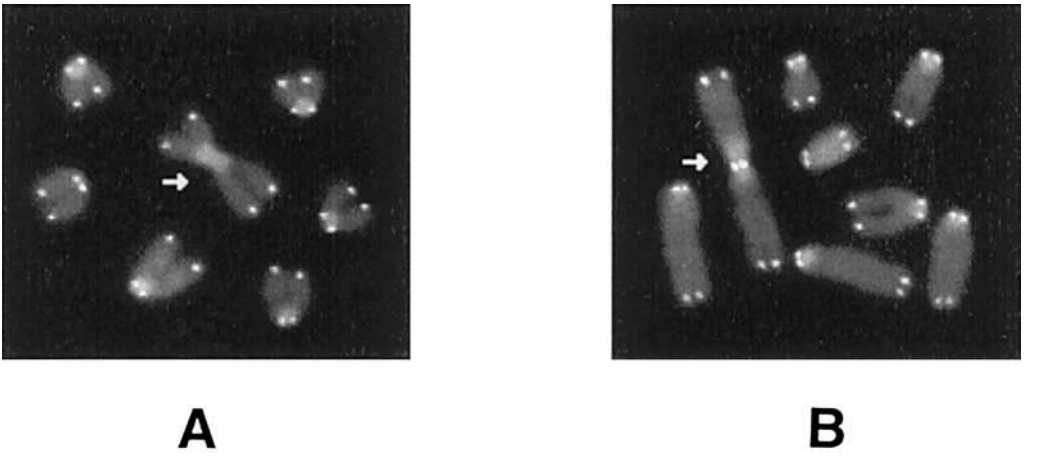


Fig. 3. Partial metaphase spreads illustrating typical mouse acrocentric chromosomes and Robertsonian translocations *(arrows).* **(A)** Repair-proficient control *without* telomere sequences at the point of fusion. **(B)** Repair-deficient mutant *with* telomere sequences present at the point of fusion.

passaging. Interestingly, these aberrations, which must be dicentrics, were not lethal to the cells possessing them, and in fact appeared to confer a growth advantage since they appeared in all immortalized cells. In contrast, SV-40 virally transformed *scid* cells restored to repair proficiency through transfection with a functional cDNA copy of the human DNA-PKcs gene, exhibited declining numbers of telomeric fusions with each successive passage until the culture became essentially free of these aberrations. Presumably, those telomeric fusions that existed in the population at the time of transfection were unstable, or cells containing them were selected against. These results demonstrate that restoration of DNA-PKcs function to *scid* cells reestablishes efficient telomeric end-capping.

The ability of Ku to bind mammalian telomeric DNA in vitro has been demonstrated *(4).* In addition, Ku has recently been localized to mammalian telomeric repeats using an in vivo crosslinking method *(51),* providing further support for a direct role of Ku as a mammalian telomeric protein. The impaired end-capping phenotype associated with DSB repair deficiency is strikingly similar to that caused by the inducible expression of a dominant negative allele of TRF2 *(109),* although it is less severe. Collectively, these results suggest that there may be at least two mechanisms mediating chromosomal end fusion. The first is a telomere length-dependent mechanism in which shortening of telomeric sequence beyond a critical value leads to loss of telomere function. The second mechanism is telomere length-independent, with telomeric fusion resulting from an inability to maintain a special protective structure at the very terminus of the chromosome and involving DNA-PK. Both mechanisms promote an unstable genetic inheritance that may contribute to the process of carcinogenesis.

Using a yeast two-hybrid screen with TRF1, a negative telomere length regulator (*see* Subheading 2.2.), an additional human telomere-associated protein, termed tankyrase, was isolated *(100).* Tankyrase has a COOH-terminal region with homology to the catalytic domain of poly(ADP-ribose)polymerase (PARP). PARP is an abundant

and highly conserved nuclear enzyme found in most eukaryotic cells that rapidly binds single- and double-stranded DNA, is activated by DNA damage (strand breaks), and catalyzes the formation of poly(ADP-ribose) onto a protein acceptor using nicotinomide adenine dinucleotide (NAD^+) as a substrate *(61).* Tankyrase has PARP-like activity with at least two specific substrates, TRF1 and tankyrase itself, and is strongly inhibited by the PARP inhibitor 3-aminobenzamide (3-AB). Modification of TRF1 and tankyrase by poly(ADP-ribosyl)ation decreases their binding affinity to telomeric DNA, and so may increase access to the telomere by telomerase and/or other end-binding proteins. Indirect immunofluorescence studies of metaphase chromosomes, probing with both anti-tankyrase and anti-TRF1 antibodies, revealed colocalization of the two signals at or near the physical ends of the chromosomes *(100),* further implicating tankyrase as a component of the human telomeric complex. In light of recent evidence that various DNA repair proteins are involved in normal telomere function, and assuming tankyrase plays a similar role to PARP's known role as a negative regulator of recombination, the presence of tankyrase at telomeres makes it a plausible candidate for involvement in suppression of inappropriate recombination activities in telomeric DNA, however a direct role has yet to be demonstrated. Chromosomal instability and telomere shortening have been reported in mice lacking PARP *(24).* In addition, it was observed that the absence of PARP did not affect the presence of telomeric single-stranded overhangs. It is also of interest to note that poly(ADP-ribosyl)ation by PARP has been shown to stimulate the protein kinase activity of DNA-PK *(92).*

What emerges from these yeast and mammalian studies is an as yet incomplete and sometimes contradictory picture that surprisingly places many DNA repair proteins at chromosomal termini where they play an unexpected yet crucial role in normal telomere maintenance and function. Further elucidation of the relationship between telomeres, DNA repair proteins, and "making ends meet" in illegitimate rejoining events will provide valuable new insight into this intriguing paradox. For example, through their contribution to efficient telomeric end capping, NHEJ repair genes help to preserve the fidelity of genetic inheritance. Whether or not other repair genes are similarly required, the molecular mechanisms through which they act, and the in vivo ramifications of ineffective end capping remain topics for future investigation. It is clear that a complete understanding of DNA repair-deficient phenotypes, and in particular how these phenotypes relate to cancer predisposition, will need to include the new role of DNA repair genes in telomere function.

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Conservation of Eukaryotic DNA Repair Mechanisms

Alan R. Lehmann and Elaine M. Taylor

1. INTRODUCTION

The fundamental importance of DNA repair for all organisms has become widely acknowledged in recent years. Evidence for the crucial role of DNA repair for the survival of all organisms comes from (1) the diversity of different repair processes; (2) the remarkable finding that about 2% of the *Escherichia coli* chromosome encodes proteins involved in DNA repair processes; (3) the extraordinary degree of evolutionary conservation of DNA repair mechanisms and proteins in all organisms. DNA repair pathways have been largely conserved from bacteria to mammals. In the vast majority of cases, the proteins that carry out these repair processes are conserved in structure and function in eukaryotes, and in some cases in bacteria as well. This conservation has been of great value in assisting our understanding of the mechanisms of DNA repair. The different strengths of working with yeasts on the one hand, which are very amenable to genetic analysis, and human cells on the other hand, which are often more amenable to biochemistry and provide relationships to human diseases, has greatly accelerated work in this area. Conclusions derived from the genetic and biochemical analysis of repair pathways in yeast can be extrapolated to human systems, and vice versa.

In this chapter we discuss the conservation of DNA repair proteins. It would be impossible to provide a comprehensive review, including all the information from the various genome projects that is now available in the sequence databases. We therefore concentrate mainly on published data on proteins from mammalian cells and the budding yeast, *Saccharomvces cerevisiae.* Where appropriate we also consider the bacterium, *E. coli,* and lessons can also be learned from the fission yeast, *Schizosaccharomyces pombe.* The latter is very distantly related to *S. cerevisiae,* so that conservation between these two yeasts is likely to extend throughout eukaryotes.

2. DNA DAMAGE REVERSAL: *PHOTOLYASES AND METHYLGUANINE METHYLTRANSFERASES*

Two DNA repair processes are unusual in that they require only a single protein that is able to reverse a specific form of damage in the DNA *in situ.* Photoreactivation, the first DNA repair process to be discovered *(57),* is brought about by the enzyme DNA

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photolyase. Photolyase binds to cyclobutane pyrimidine dimers (CPDs) in the dark and reforms the monopyrimidines on exposure to long wavelength UV or visible light. In an extensive study of photolyases from different organisms, Yasui and coworkers *(196)* were able to delineate two distinct classes of photolyases, with little sequence similarity between the two classes. Class I encompasses the enzymes from eubacteria, halobacteria and lower eukaryotes (including *S. cerevisiae*), whereas class II occur in methanobacteria and higher eukaryotes (including birds, fish, and nonplacental mammals). Interestingly, no photolyase has been identified in *S. pombe,* nor is there convincing evidence for photolyase in placental mammals. This interesting and unusual evolutionary development is quite different from that found in other repair pathways discussed in the following sections.

Within the last few years a gene encoding a photolyase that reverses the 6-4 photoproduct rather than the CPD has been identified in *Drosophila melanogaster (86,176).* This photolyase has 20–22% identity with the class I CPD photolyases, and 22–24% identity with blue-light photoreceptors, which appear also to belong to the same superfamily. Two human homologs of this class of genes have been isolated recently *(175,181),* but they appear to encode blue-light receptors rather than active photolyases. This family of genes and their activities has been reviewed by Yasui and Eker in Volume 2, Chapter 2, of this series.

The second damage reversal process is the demethylation of the highly mutagenic methylated base O^6 methylguanine by O^6 methylguanine DNA-methyltransferase (MGMT). The mechanism of action is the same in all organisms, namely the removal of the methyl group onto a cysteine residue in the active site of the protein, the resulting S-methylcysteine causing irreversible inactivation of the protein activity. In *E. coli* there are two proteins with MGMT activity. The Ada protein is highly inducible by methylating agents and has two methyltransferase activities in separate domains of the protein. The N-terminal domain has an activity which removes methyl groups from methylphosphotriesters, whereas the C-terminal domain contains the MGMT activity. The second (constitutive) *E. coli* protein Ogt, and eukaryotic homologs, contain only the latter activity. Both the yeast and human homologs were cloned by their abilities to correct the sensitivity to methylating agents of *E. coli* MGMT mutants *(72,194).* Human MGMT is about 28% identical to both Ogt and the C-terminal domain of Ada, with much higher conservation in the extreme C-terminus, which contains the active site cysteine residue (e.g. refs. *145,170*). Likewise the yeast Mgt1p is 43% identical to the *E. coli* proteins and 34% identical to the human protein over the C-terminal 88 amino acids *(194).*

3. BASE EXCISION REPAIR

DNA base damage can occur spontaneously (e.g., by hydrolytic deamination of cytosine and 5-methylcytosine), as an undesirable byproduct of cellular oxidative processes, or as a result of reactions with simple alkylating agents or ionizing radiation. This type of damage is corrected by the base excision repair (BER) pathway (*see* ref. *92* for a recent review). BER is initiated by a DNA glycosylase, which cleaves the glycosylic bond between the modified base and the sugar-phosphate backbone. DNA glycosylase action thereby excises the damaged moiety as a free base, and generates an abasic site within the DNA. Some DNA glycosylases are specific for a particular substrate (e.g.,

Table 1
Sequence Homology of Proteins Involved in BER

Protein	Activity	% Identity *S. cerevisiae*/man	% Identity *E. coli*/man	Functional complementation man/*E. coli*	Reference
hUDG1	Uracil-DNA glycosylase	40	—	Yes	*(92,124)*
hTDG	Thymine-DNA glycosylase	—	29	No report in literature	
hOGG1	8-oxoguanine-DNA glycosylase	38	—	Yes	*(140,144)*
hMYH	Adenine DNA-glycosylase	(31% *S. pombe*/man)	41	No report in literature	*(102,163)*
MPG	3-methyladenine-DNA glycosylase	—	—	Yes	*(31,121, 148)*
hNTH1	Endonuclease III	50% (over about half the protein)	22.5	*nth* mutants have no phenotype	*(8,43)*
HAP1 (APE1)	AP endonuclease	21	28	yes	*(14,38,79, 142)*

uracil), whereas others are able to recognise several different types of damaged DNA base *(92).* The resulting abasic site is converted to a single nucleotide gap by the action of an AP endonuclease, which cleaves the phosphodiester backbone, and a deoxyribosephosphodiesterase (dRpase), which removes the deoxyribose phosphate from the 5′ end of the cleaved strand. Short-patch gap-filling by a DNA polymerase and DNA ligase completes the repair process.

In recent years many of the genes required for BER have been identified in *E. coli,* yeast, and mammalian cells, revealing a high degree of evolutionary conservation (Table 1). Several of the eukaryotic genes were cloned by complementation of *E. coli* BER mutants. For example, the *S. cerevisiae OGG1* gene, which encodes an 8-oxoguanine DNA glycosylase, was cloned by functional complementation of an *E. coli mutM* mutant *(180).* In this case, Ogg1p can functionally substitute for the MutM glycosylase despite sharing little sequence similarity. A human homolog of *OGG1, hOGG1* (38% identical to the yeast protein at the amino acid level), is able to complement the mutator phenotype of both the yeast *ogg1* and *E. coli mutM* mutants *(140,144).*

The MutY protein of *E. coli* encodes an adenine glycosylase that removes adenines from A/G or A/8-oxoguanine mispairs. Although homologs have been isolated both from human sources with 41% identity, and from *S. pombe* with 28% identity to MutY, no homolog has been found in the *S. cerevisiae* genome *(102,163).* Eukaryotic homologs of the *E. coli* uracil DNA glycosylases *(ung and mug) (62,162),* 3-methyladenine DNA glycosylase *(alkA) (31,121,148)* and endonuclease III *(nth) (8,43)* have also been cloned and characterized (see Table 1). The human uracil glycosylase is able to complement the phenotype of *E. coli ung* mutants, and human MPG protein (AlkA homolog) complements the alkylation sensitivity of *E. coli alkA tag* mutants. (Complementation of *mutY* mutants by the corresponding human genes has not been reported,

and *nth* mutants of *E. coli* do not have a phenotype that can be analyzed in a complementation assay).

In *E. coli* there are two AP endonucleases. The major activity is associated with exonuclease III (Xth), the minor activity being endonuclease IV (Nfo). In human cells, the major AP endonuclease activity is encoded by the *HAP1/APE1* gene. HAP1 endonuclease shares 28% identity with *E. coli* Xth, and is able to complement some of the mutant phenotypes of the *E. coli xth* mutant *(38,142).* A structural homolog, designated *APN2* or *ETH1,* was also identified recently in yeast, but the major AP endonuclease in this organism is the product of the *APN1* gene. Apn1p shares 41% identity with the minor *E. coli* AP endonuclease, Nfo *(139).* Thus the major AP endonuclease in yeast is the homolog of the minor AP endonuclease in *E. coli* and vice versa.

Although the general strategy of BER is highly conserved from bacteria to humans, the later stages of this repair process seem to differ in higher eukaryotes. For example in mammals, in contrast to *E. coli,* a single enzyme, DNA polymerase β, performs both the dRpase and gap filling functions *(105,164).* Moreover, no yeast counterparts have been identified for either DNA ligase III or XRCC1, which are together thought to provide the major DNA joining activity required for the completion of BER in mammals *(21,174).*

4. MISMATCH REPAIR (MMR)

DNA mismatches result from errors during DNA replication. In order to ensure that such mismatches are repaired in the correct orientation, the cell must have a mechanism for distinguishing between parental and daughter strands. In *E. coli* this is effected by methylation at the 6-position of adenines in GATC sequences by the Dam methylase, a process that occurs subsequent to synthesis of the daughter strands (*see* Vol. 1, Chapter 11). The initial steps of MMR are the recognition of the mismatch by the MutS protein, which then recruits the MutL followed by the MutH protein. The latter cuts the DNA opposite the nearest hemi-methylated GATC sequence, and this incision on the daughter strand initiates a long patch excision repair process. Methylation of the parental strands is one of the few repair-related mechanisms that is not conserved in eukaryotes. Thus there are no known eukaryotic homologs of either Dam or MutH. In *S. cerevisiae* there are, however, six MutS and four MutL homologs. Three of the former, Msh2p, Msh3p, and Msh6p, and three of the latter, Mlh1p, Mlh3p and Pms1p have been implicated in MMR. The role of MutS in *E. coli* is taken over in yeast by two separate heterodimers, namely Msh2p/Msh3p and Msh2p/Msh6p, which have specificities for different types of mismatches, the former having preference for single-base mispairs, the latter for small insertion/deletion mismatches. Similarly the function of MutL is for the most part substituted with heterodimers of Mlh1p/Pms1p. Recent work has suggested however, that a proportion of the repair of specific insertion/deletion mispairs by the Msh3p-dependent MMR pathway uses a heterodimeric Mlh1p/Mlh3p complex in place of the Mlh1p/Pms1p *(54).*

All the yeast MMR proteins proteins have human homologs, whose importance has been demonstrated by their association with familial colon cancer. Many patients with hereditary nonpolyposis colon carcinoma (HNPCC) are associated with defects in hMSH2, hMLH1, and hPMS2 *(51,134)* and there is evidence in a few cases for association with hPMS1 *(119),* and hMSH6 *(2).* The phylogenetic relationships between the

Table 2
Sequence Homology of Proteins Involved in MMR

Protein	Activity	% Identity *S. cerevisiae*/man	Reference
hMSH2	Mismatch recognition	40	*(51)*
hMSH3	Mismatch recognition	30	*(59,118)*
hMSH6	Mismatch recognition	29	*(129)*
hMLH1	Binding to Msh2 complex	34	*(130)*
hPMS1	Binding to Msh2 complex	20 (Mlh3p)	*(54)*
hPMS2	Binding to Msh2 complex	32 (Pms1p)	*(119)*

different homologs are discussed in detail by Crouse in Vol. 1, Chapter 19 and by Fishel and Wilson *(52).* The sequence identities between the *E. coli,* yeast, and human proteins that have been implicated in MMR are presented in Table 2. (Note the confusing nomenclature: hPMS2 appears to be the homolog of yeast Pms1p, and hPMS1 appears to be the homolog of yeast Mlh3p.)

5. NUCLEOTIDE EXCISION REPAIR (NER)

Nucleotide excision repair (NER) is a versatile DNA-repair pathway that can act on a wide variety of DNA alterations. It is particularly effective on bulky, helix-distorting lesions, such as UV-induced DNA photoproducts. Relative to BER, NER is a complex process, requiring the concerted action of many proteins, in order to recognize the lesion, open up the DNA structure around the site of the damage and excise the damage-containing strand (*see* ref. *192* for a review). In humans, defects in NER are associated with the inherited syndromes xeroderma pigmentosum (XP), trichothiodystrophy (TTD) and Cockayne Syndrome (CS).

Most of the genes required for NER have been cloned from yeast and from humans, and biochemical studies on their encoded products have identified the functional activities associated with many of these proteins (*see* Table 3 and Volume 2, Chapter 18). Most were cloned by complementation of radiation sensitive mutants but some (e.g., XPF, ref. *161*) have been cloned on the basis of sequence homology with yeast genes. Conversely, when the human *XPB/ERCC3* gene was cloned by complementation of a UV-sensitive rodent mutant, no yeast homolog was known. The homologous *RAD25* was subsequently isolated by hybridization with *XPB* cDNA *(131).* A striking feature of all the NER genes cloned and sequenced to date is the high degree of structural similarity which exists between proteins from such evolutionarily distant organisms. Despite this structural homology, interspecies complementation of NER defects is rare, reflecting the multiplicity of protein-protein interactions required for this complex repair process. In BER, where a number of enzymes act sequentially at the site of damage and little requirement for protein-protein contact might be anticipated, only those parts of the BER enzyme important for catalytic activity need to be conserved in order for cross-species complementation to be effective. In contrast, NER demands the concerted action of a large number of proteins, each of which interacts with other members of the repair complex. Functional complementation of NER defects therefore requires that protein structure must be conserved, not only in those regions important for catalytic function, but also in areas required for protein-protein interactions.

Table 3
Sequence Homology of Proteins Involved in NER

Human Protein	Protein activity	% Identity *S. cerevisiae*/man[a]	% Identity *S. pombe/S. cerevisiae*[b]	Reference
XPA	Damage-specific DNA-binding protein	27 (Rad14p)		*(9)*
XPC	Damage-specific DNA-binding protein	23(Rad4p)		*(93)*
HR23B	Associated with XPC	32 (Rad23p)		*(104)*
XPG (ERCC5)	Damage-specific 3′ endonuclease	24 (Rad2p)	33 (Rad13p)	*(25,154)*
XPF (ERCC4)	Damage-specific 5′ nuclease subunit	26 (Rad1p)	30 (Rad16p)	*(24,161)*
ERCC1	Damage-specific 5′ nuclease subunit	35 (C-terminal half) (Rad10p)	39 (C-terminal half) (Swi10p)	*(143,182)*
XPD (ERCC2)	5′-3′ helicase; subunit of TFIIH	52 (Rad3p)	65 (Rad15p)	*(116,187)*
XPB (ERCC3)	3′-5′ helicase; subunit of TFIIH	55 (Ss12p/Rad25p)		*(131)*
p62	TFIIH subunit	26(Tfb1p)		*(48)*
p52	TFIIH subunit	40 (Tfb2p)		
p44	TFIIH subunit	42 (Ss11p)		
p34	TFIIH subunit	33 (Tfb4p)		

[a] *S. cerevisiae* protein in parenthesis.
[b] *S. pombe* protein in parentheses.

A summary of the functional activities of eukaryotic NER genes cloned to date is presented in Table 3. The XPC protein shows some homology to *S. cerevisiae* Rad4p *(93),* and both interact with a second protein (HR23B in humans and Rad23p in yeast), which is evolutionarily conserved *(68,104).* The XPC-HR23B complex has been recently shown to act at the first step in NER, namely damage recognition (165). This complex is then displaced by XPA. The human XPA protein and its *S. cerevisiae* homolog Rad14p are DNA binding proteins, both of which contain a zinc finger motif *(7,69).* TFIIH is a multiprotein complex that functions in initiation of RNA polymerase II-mediated transcription, and is absolutely required for NER in both yeast and mammalian cells *(169).* Genes for all nine subunits of TFIIH have now been described for both humans and yeast, revealing an extraordinary degree of evolutionary conservation *(48).* In yeast five of these nine components (Rad3p, Rad25p/Ss12p, Ss11p, Tfb1p, and Tfb2p) have thus far been shown definitively to play a role in NER *(48,186),* and it is likely that the whole TFIIH complex operates in both processes, albeit in different forms *(169).* Perhaps the best characterized of the TFIIH subunits are the products of the *RAD25* and *RAD3* genes. Rad25p and its human counterpart XPB function as 3′–5′ DNA helicases, whereas Rad3p and its homologs (XPD in humans and Rad15p in *S. pombe*) are 5′-3′ DNA helicases *(77).* The ATP-dependent helicase activities of XPB and XPD are required for the limited opening of the DNA duplex around the site of a DNA lesion *(47).* The formation of this open DNA intermediate is a prerequisite for dual incision by the NER nucleases *(46).*

A further interesting aspect reflecting evolutionary conservation is the frequency of mutations in *XPD/RAD3* compared to *XPB/RAD25.* In mutant screens for UV-sensitive mutants of *S. cerevisiae,* many *rad3* alleles were isolated. Likewise there are a substantial number of XP and TTD patients mutated at different sites in *XPD (172).* In contrast mutations in *RAD25* were never detected in mutant screens and there are only three known families with mutations in *XPB.* This shows that in both organisms Rad3p/XPD is tolerant of small alterations, which still permit TFIIH to function in transcription. In contrast Rad25p/XPB can only tolerate very few alterations without destroying TFIIH transcription function with resulting fatal consequences.

Two structure-specific nuclease activities are associated with eukaryotic NER, one of which cleaves the DNA on the 3′-side of the damage, the other on the 5′ side. The 3′-endonuclease is encoded by *RAD2* in *S. cerevisiae* and *XPG* in humans *(10,122).* XPG, Rad2p, and *S. pombe* Rad13p all share a high level of sequence identity over two functionally important regions close to the N- and C-termini, respectively *(25,54).* Moreover, in a rare instance of interspecies functional complementation, the *S. cerevisiae RAD2* gene is partially able to rescue the UV sensitivity of a *S. pombe rad13* mutant *(107).* The endonuclease activity which cleaves on the 5′-side of a DNA lesion is associated with a heterodimeric complex, comprised of the Rad1p and Rad10p proteins in *S. cerevisiae* and ERCC1 and XPF from humans *(11,161).* The homologous gene products in *S. pombe,* Rad16p and Swi10p, have also been shown to interact and presumably fulfil the same role in fission yeast *(24).* Curiously, the domains involved in the interaction of ERCC1 and XPF appear to be different from those that modulate Rad1p/Rad10p interactions *(37).*

6. REPAIR OF DSBs

Although we still have relatively little understanding of the enzymatic mechanisms of repair of DSBs, over the last five years there have been tremendous advances in our

understanding of the molecular genetics of this process. Two major pathways have been delineated. The first, involving homologous recombination (HR), appears to be the sole mechanism for repairing DSBs in *E. coli.* In yeast, it is the major pathway, but it plays a less important role in mammalian cells. Conversely, the second pathway, nonhomologous end-joining (NHEJ) has not been found in *E. coli,* plays a minor role in yeast, but is a major pathway for repairing γ-ray induced DSBs in mammals.

6.1. Recombination Repair

The key protein mediating homologous recombination in bacteria is RecA, which forms nucleoprotein filaments and promotes pairing of homologous DNA sequences and strand transfer *(58).* In yeast, repair of DSBs by HR requires the products of the *RAD51, 52, 54, 55* and *57* genes, and the ability to repair DSBs is severely compromised in mutants of these genes. There is evidence that the products of these genes interact to form a multiprotein complex *(33,73,109,166,167).* Rad51p is homologous to RecA. Although the sequence homology is not particularly dramatic (30% over the C-terminal half of the protein, ref. *159*), Rad51p forms nucleoprotein filaments with DNA that are structurally almost indistinguishable from RecA filaments *(123).* The sequence conservation of this group of proteins in eukaryotes is very high (Table 4) and the genes from higher organisms that operate in this pathway have been cloned using techniques based on sequence homology. Homologs of Rad51p have been isolated from fission yeast, chicken and mammalian species, and the degree of sequence (and presumably structural) identity is very striking *(158).* The human protein, like RecA, promotes homologous pairing and strand transfer, but the polarity of the transfer is opposite to that mediated by RecA *(12).* Two other yeast proteins, Rad55p and Rad57p, which promote strand exchange mediated by Rad51p *(167),* are in the same protein family as Rad51 *(82,101).* At least five additional mammalian members of this family have also been recently identified (*see* Table 5). Two of these, *XRCC2* and *XRCC3* were isolated by their ability to correct the mitomycin C (MMC) sensitivity of mutants that are sensitive to ionizing radiation and cross-linking agents *(27,97).* These are therefore implicated in recombination repair. The other family members were identified by screening of the expressed-sequence-tag (EST) databases. Their functions have yet to be elucidated.

Rad54p, which interacts with Rad51p *(33),* also shows a high degree of sequence conservation. Curiously, however, Rad52p, another key protein in HR in yeast, which stimulates pairing mediated by Rad51p *(166),* is much less well conserved than Rad51p or Rad54p *(112,125)* (*see* Table 4). Cells from a recently constructed *rad52* knockout mouse are not sensitive to ionizing radiation at all *(141).* The reason for this is not yet clear, and does not rule out a role for Rad52p in repair of DSBs in mammalian cells. There may be more than one homologous protein with overlapping functions, and it has been shown that overexpression of human *RAD52* confers enhanced radioresistance to cultured monkey cells *(132).* Two human homologues of Rad54p have been identified *(75,81,114)*

Two genes associated with familial breast cancer, *BRCA1* and *BRCA2* have been implicated in repair of ionizing radiation damage. Evidence has been produced that both BRCA1 and BRCA2 interact with hRAD51 (e.g., *see* refs. *156,157*), and that *BRCA2*-defective cells are deficient in their ability to repair DSBs *(1,35).* These results implicate BRCA1 and BRCA2 in HR, but further work is needed to define their precise roles. No homologs of these genes have been identified in lower eukaryotes, implying a

Table 4
Sequence Homology of Key Proteins Involved in HR

Human protein	Activity	% Identity *S. cerevisiae*/ man	% Identity *S. pombe*/ *S. cerevisiae*	Reference
hRAD51	Strand exchange	69	67	*(113,158)*
hRAD52	Cofactor for RAD51	30	27	*(112,125)*
hRAD54		48	51	*(81,114)*
hRAD50	Interacts with hMre11	28	35	*(40)*(E Hartsuiker, personal communication)
hMRE11	3′–5′ Exonuclease	34	50 (over 500 aa)	*(137,171)*
NBS1 (Nibrin)	Interacts with hMre11	28 (over 115 aa) with Xrs2p		*(23,183)*

Table 5
RecA/Rad51 Homologs in Humans

Rad51p homolog	Isolation/function	% Identity to *S. cerevisiae* gene	Reference
HsRAD51	Hybridization	67% to Rad51p	*(158)*
XRCC2	Correction of irs1 mutant	20–25% to Rad51p, 55p, 57p	*(27,97)*
XRCC3	Correction of irs1SF mutant	28–31% to Rad51p, 57p	*(97)*
HsRAD51B/ HREC2/ RAD51H2	EST database	30–31% to Rad51p, 57p	*(3,26)*
HsRAD51H3/ RAD51D	EST database	25–27% to Rad51p, 55p, 57p	*(26,138)*
RAD51C	EST database	31% to Rad51p	*(41)*

specific role for the proteins in multicellular organisms. Despite the absence of obvious homologs of BRCA1 and BRCA2 in simpler organisms, BRCA1 contains domains designated BRCT domains, which have been found in many proteins involved in DNA repair and DNA damage responses *(17,22).* This motif is of unknown function and comprises about 100 amino acids in five conserved blocks.

Three other yeast proteins, which are known to form a stable complex and to be involved in repair of radiation damage, are the products of the *RAD50, MRE11,* and *XRS2* genes *(80).* They are involved both in the formation and rejoining of breaks during meiosis, and there is evidence that they are involved in both HR and NHEJ in *S. cerevisiae (20,108,177).* Human homologs of *RAD50* and *MRE11* have been isolated *(40,137).* The protein complex containing human MRE11 and RAD50 was isolated recently and found to contain other subunits. One of these had limited sequence identity to Xrs2p at the N-terminus, and is likely to be its functional homolog. Excitingly this protein was found to be the product of the gene defective in the Nijmegen Breakage Syndrome *(23,183).* This disorder has clinical and cellular characteristics related to

Table 6
Sequence Homology of Proteins Implicated in NHEJ

Human protein	Protein activity	% Identity *S. cerevisiae*/man	Reference
Ku70	DNA end-binding	22 (over about 50% of the protein)	*(49)*
Ku86	DNA end-binding	20	*(18,108)*
DNA-PK_{CS}	Protein kinase	—	*(71)*
Ligase IV	Break joining	24	*(153,173, 191)*
XRCC4	Activator of ligase IV	22	*(74,94)*

ataxia-telangiectasia (A-T) (*see* Section 7.). At the time of writing, there is no evidence for the involvement of these human homologs in NHEJ in humans.

Although HR plays a role in the response of mammalian cells to radiation, as shown by the radiation-sensitivity of cells derived from the *RAD54* knockout mice *(45),* this sensitivity is not very drastic. This suggests that there are alternative routes for repairing DSBs in mammalian cells.

6.2. Nonhomologous End-Joining

A major pathway for repair of DSBs in mammals appears to be NHEJ, as shown by the very marked radiosensitivity of mutant cells defective in this pathway (reviewed in ref. *78*). Several of the proteins involved in NHEJ have recently been identified and characterized. DNA-dependent protein kinase is absolutely required for this process. It is composed of the 70 and 86 kDa subunits of the DNA-end binding Ku protein, and the 460 kDa catalytic subunit (DNA-PK_{CS}), which is recruited when Ku binds to DNA ends (reviewed in ref. *78*). Homologs of the two Ku subunits have been identified in yeast (*see* Table 6). The degree of sequence identity to the mammalian proteins is quite low. Mutants in yeast *KU70* or *KU86* are barely sensitive to ionizing radiation, because of the major role played by HR *(160).* However, in a *rad52* background in which HR can no longer take place, deletion of the *KU* genes results in a substantial further sensitization to radiation *(160).* Evidence for the involvement of Ku in NHEJ in yeast has come from the use of plasmid systems, in which plasmids cut with restriction enzymes are transformed into yeast cells and are substrates for NHEJ. Rejoining of these breaks requires intact yeast *KU* genes *(18,19,108,178).* Despite both the sequence and the functional similarity between yeast and mammalian Ku proteins, there is no evidence either biochemically or from the complete sequence of the yeast genome, that a homolog of DNA-PK_{CS} exists in yeast. In mammals, defects in Ku subunits or in DNA-PK_{CS} result in similar radiation sensitivities, suggesting that the whole complex participates in NHEJ in mammals. This is therefore a curious divergence between yeast and humans, which awaits a satisfactory explanation.

Strong evidence has been produced to show that the joining step of NHEJ is mediated by DNA ligase IV, which forms a tight complex with the XRCC4 protein *(36,63).* The latter had been shown by genetic studies to be involved in NHEJ. This work led to the identification of a homolog of DNA ligase IV in the yeast genome and the demonstration of its involvement in NHEJ in yeast *(153,173,191).* An XRCC4 homolog, designated Lif1p, which interacts strongly with yeast DNA ligase IV has also been identified recently *(74).*

Thus in the NHEJ pathway, we have the interesting finding that the process is conserved from yeast to man and many of the proteins are structurally conserved, but DNA-PK_{CS}, a key protein essential for NHEJ in mammalian cells, does not have an identified structural homolog in yeast.

7. DNA DAMAGE CHECKPOINTS

In addition to the DNA repair processes discussed earlier, eukaryotic cells also possess regulatory mechanisms known as checkpoints, which function to arrest cell-cycle progression in response to DNA damage. These cell cycle delays provide time for the completion of DNA repair before replication or mitotic segregation take place. Failure to respond to DNA damage in this way can lead to an increase in genomic instability, and checkpoint defects in humans are often associated with carcinogenesis.

Much of our current knowledge regarding DNA damage checkpoint control has been derived from genetic studies using the yeasts *S. cerevisiae* and *S. pombe* (reviewed in refs. *29,99,120,188*). Evidence from these organisms suggests that specific protein complexes detect DNA damage and activate a signal transduction pathway to transmit inhibitory signals to the cell cycle machinery. Many of the genes required for DNA damage checkpoint functions have been identified in both budding and fission yeast, and more recently a number of their human homologs have been cloned on the basis of sequence similarity (*see* Table 7). The degree of structural similarity that is evident between checkpoint proteins from different species suggests that DNA-damage checkpoint mechanisms have, like DNA-repair processes, been highly conserved throughout evolution.

In *S. pombe* the products of six genes *(rad1, rad3, rad9, rad17, rad26 and hus1)* required for checkpoint function *(4)* form three complexes, which comprise the upstream components of the checkpoint response. The *S. pombe* Rad3p/Rad26p complex *(42)* is required for all checkpoint functions and is probably involved in the recognition step of the checkpoint response. Homologs of Rad3p have been identified in several organisms, and in *S. cerevisiae* a homolog of Rad26p, designated Ddc2p, has been found with very similar function to Rad26p, but relatively poorly conserved at the sequence level *(126).* As yet no homolog has been identified in the mammalian EST databases. Rad3p and its homologs in *S. cerevisae* (Mec1p) and humans (ATR) are members of a conserved superfamily of phosphatidylinositol 3-kinase-related proteins (PIK-like kinases) *(15,32,84,189).* Despite their structural similarity to lipid kinases, no associated lipid kinase activity has been found for any of these proteins. Instead, protein kinase activity has been demonstrated for both Rad3p and ATR *(15,85).* This kinase activity is essential for their function in checkpoint control *(15,193).* DNA damage-induced phosphorylation of many of the proteins that participate in the DNA-damage checkpoint is dependent on Rad3p/Mec1p function *(96,99,185).* Another closely related member of the PIK-like kinase family is encoded by *ATM,* the gene that is mutated in the inherited, cancer-prone disorder ataxia-telangiectasia (A-T) *(152).* Cells derived from A-T patients are highly sensitive to ionizing radiation and are defective in G1/S, intra-S, and G2/M checkpoints following ionizing radiation *(13,83,128).* ATM shares significant sequence similarity with Rad3p and Mec1p (21% and 19% identity to Rad3 and Mec1 respectively), but it is actually more closely related to the products of the Tel1p proteins in *S. cerevisiae* and *S. pombe* (*see* Table 7) *(65,110,117).* Surprisingly, *S. cerevisiae tel1* mutants do not exhibit a checkpoint defect, although there does seem to

Table 7
Sequence Homology of DNA Damage Checkpoint Proteins

S. pombe protein	*S. cerevisiae* protein	Human protein	% Identity *S. pombe/ S. cerevisiae*	% Identity *S. pombe*/man	Protein properties	Reference
Rad1p	Rad17p	hRAD1	23	27	Potential nuclease Limited homology to PCNA	*(56,103,168,179)*
Rad3p	Mec1p	ATR	23	26	PI3-kinase related	*(15,32,84,189)*
Rad9p	Ddc1p	hRAD9	21	25	Limited homology to PCNA	*(95,100,115)*
Rad17p	Rad24p	hRAD17	20	49	Limited homology to RF-C	*(66,133)*
Rad26p	Ddc2p		14		Interacts with Rad3p	*(4,126)*
Hus1p	Mec3p	hHUS1	16	30	Limited homology to PCNA	*(89,90)*
Chk1p	Chk1p	hCHK1	32	29	Protein kinase	*(53,150)*
Rad24p and Rad25p	Bmh1p and Bmh2p	14-3-3 proteins	71	55	14-3-3 proteins	*(55)*
Crb2p/Rhp9p	Rad9p		26		BRCT domain protein	*(146,155,190)*
Cds1p	Rad53p	hCHK2	35	30	Protein kinase	*(5,16,106,111)*
Rad4p/Cut5p	Dpb11p	database	27			*(6,50,147)*
Cut2p	Pds1p		20		Anaphase inhibitor	*(60,195)*
Te11p	Te11p	ATM	22	23	PI3-kinase related	*(65,110,117,152)*
		p53			Transcription factor	*(197)*
		p21			Cdk inhibitor	*(67,70)*

be a degree of functional overlap between Mec1p and Tel1p, because Tel1p overexpression can partially complement some of the radiation sensitivity associated with a *mec1* mutant *(110).*

The *S. pombe* proteins Rad1p, Rad9p, and Hus1p all have some sequence similarity to PCNA *(30).* There is direct evidence for physical interactions between these three proteins *(30,89),* their *S. cerevisiae* homologs (Rad17p, Ddc1p, and Mec3p respectively) *(88,127),* and their human homologs *(184).* Furthermore, both hRAD1 and hRAD9 can partially rescue the checkpoint defects of the respective *S. pombe* mutants, implying functional as well as structural conservation in the DNA-damage checkpoint *(56,95).* It has been suggested that Rad1p, Rad9p, and Hus1p might form a heterotrimeric ring with a similar structure to the homotrimer formed by PCNA.

Both *S. pombe* Rad17p and its homolog in *S. cerevisiae,* Rad24p, show some sequence similarity to the components of replication factor C *(66).* A physical interaction between Rad24p and the four smaller components of replication factor C has been demonstrated in *S. cerevisiae (64).*

Although the DNA damage-sensing aspects of checkpoint control seem to be highly conserved throughout eukaryotes, the downstream signal amplification and cell-cycle responses differ somewhat between organisms. For example, in *S. cerevisiae* a major target of the DNA-damage checkpoint is the anaphase inhibitor Pds1p, which becomes phosphorylated after DNA-damage in a Mec1p-dependent manner *(34).* Pds1p is absolutely required to block cell-cycle progression in mitosis (at the metaphase-anaphase transition), following DNA damage *(195).* The fission yeast homolog of Pds1p, Cut2p, is also required to prevent the premature separation of sister chromatids during the normal cell cycle, but is apparently not a major target of the DNA-damage checkpoint *(60).* Instead, in fission yeast and in human cells, DNA damage leads to cell cycle arrest prior to the onset of mitosis, at G2/M. In *S. pombe* this arrest operates through a Chk1p protein kinase-dependent mechanism, which serves to maintain an inhibitory tyrosine phosphorylation on the cell-cycle kinase Cdc2p *(61).* Mammalian homologs of Chk1p have been identified and characterized, and they appear to operate in a similar manner to *S. pombe* Chk1p *(98,136,150).*

The *S. cerevisiae* protein kinase Rad53p is a major downstream target of Mec1p in the checkpoint response to DNA damage *(149),* and is required for G2 arrest. Rad53p is also required for the intra-S checkpoint in *S. cerevisiae* by arresting DNA replication in response to damage and preventing the firing of late origins of replication, as well as for the transcriptional induction of a number of DNA repair genes *(5,135,151).* The fission yeast homolog of Rad53p, Cds1p, functions to inhibit DNA replication when DNA damage is incurred, but does not appear to play a major role in inhibiting mitosis *(96).* Thus Rad53p and Cds1p are clearly structural and functional homologs and both require Mec1p/Rad3p for activation, but Rad53p is an essential component of all DNA damage checkpoints, whereas Cds1p is not. A human homolog of Rad53p/Cds1p has been identified and designated hChk2. Its activity is dependent on the ATM protein kinase *(16,106).* hChk2 is required to maintain the G2 checkpoint after ionizing radiation *(76).*

The apparent conservation of G2/M checkpoint proteins means that the genetic analysis of DNA-damage checkpoints in yeast is likely to be very informative with regard to higher eukaryotic-checkpoint control. To date, much of the work regarding

DNA-damage checkpoints in mammalian cells has focused on the G1/S cell-cycle transition and its regulation by the p53 tumor-suppressor protein. DNA damage results in the stabilization of p53. This upregulation of p53 is dependent on ATM, ATR, and Chk2 *(28).* Depending on the cell type and the nature of the damaging agent, p53 can have either of two downstream effects. In some cell types, p53 upregulates the cyclin-dependent kinase (CDK) inhibitor p21 *(44).* p21 binds, in multiple copies, to several cyclin-CDK complexes (e.g., cyclin D1-Cdk4, cyclin E-Cdk2, cyclin A-Cdk2, cyclin A-Cdc2) and in so doing prevents cells from exiting G1 *(67,70).* In cells such as human fibroblasts this G1 arrest is permanent *(39).* In other cell types upregulation of p53 results in cell death by apoptosis (reviewed in ref. *87*). It is thus likely that the principal function of p53 is not to assist in cell survival by allowing time for repair of DNA damage during G1 arrest. Its function is rather to remove from the cycling population those cells that have received substantial amounts of genetic damage, thereby protecting the whole organism. This protective function is only likely to be relevant to multicellular organisms, consistent with the fact that no p53 homologs have been identified in single cell organisms.

8. CONCLUSIONS

The high degree of evolutionary conservation of proteins involved in different DNA repair processes attests to their crucial importance in all organisms. The differences in the nature and conservation of these repair processes highlight different mechanisms and constraints. Of the seven processes discussed above, four (methyltransferases, BER, MMR, and HR) are conserved both structurally and functionally from *E. coli* to humans, although in MMR and HR, there is an increasing degree of complexity as we go up the evolutionary ladder. This is exemplified by the six eukaryotic homologs of the MMR protein, MutS, and in HR, the three RecA homologs in yeast and at least six in humans. With BER, although sequence conservation is not particularly striking, in many cases the genes from one organism can complement corresponding mutants in other organisms. This is consistent with BER being a sequential process of different enzymes acting on DNA substrates. With NER, functional complementation is rare, presumably because of the involvement of multiprotein complexes. It is surprising therefore, that several instances of partial complementation of yeast cell-cycle checkpoint mutants by homologous human genes have been reported. Because checkpoint proteins also appear to act in multiprotein complexes, functional complementation would not have been anticipated.

In NER, MMR, and HR every component has a sequence homolog conserved between yeast and humans, whereas in NHEJ a crucial protein in mammals does not have a sequence homolog in yeast. It should be noted, however, that there are increasing numbers of examples of proteins that have no discernible sequence similarity, yet when 3D stuctures have been solved, the structures of the proteins are almost superimposable. This is exemplified by the sliding-clamp proteins that are required to maintain the processivity of DNA polymerases in pro- and eukaryotes. In the latter case, this function is performed by PCNA, which forms a trimeric ring around the DNA, clamping DNA polymerase δ to the DNA, whereas in the former it is the β subunit of DNA polymerase III that performs this function as a dimer. The structures of the two rings are strikingly similar even though there is no significant sequence similarity *(91).* Thus, in instances

where there are no obvious sequence homologs between two organisms, the possibility of structural and functional homology is not excluded.

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